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# THE JOURNAL OF HYGIENE

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# THE TOXICITY OF ATMOSPHERES CONTAINING HYDROCYANIC ACID GAS.

By JOSEPH BARCROFT.

(From the War Department, Experimental Station.)

(With 23 Text-figures.)

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## I. INTRODUCTION.

THE lethal dose of an ordinary pharmacological preparation is expressed in a simple statement of quantity. The lethal dose of a poisonous gas cannot as a rule be so expressed. The majority of such gases are rapidly destroyed in the body, and the building up of a lethal concentration in some critical portion of the organism is a race between the rate at which the gas is inhaled and the rate at which it is destroyed. Even in the cases of such gases as arsenuretted hydrogen which are destroyed only very slowly, and which are therefore cumulative poisons, the attainment of a lethal concentration in the body is a function not only of the concentration of the gas in the atmosphere, but of the time during which that atmosphere is breathed.

As the fatal dose of a poisonous gas is best expressed by a curve which relates the concentration of the gas in the atmosphere to the length of time for which it is inhaled, if this curve could be represented by an equation (as in some cases it approximately can be), that equation would be the statement of the dose. During the great war it was the universal practice among the belligerent nations to state the toxicity of gases by concentration-time curves.

A consideration of the toxicity of hydrocyanic gas therefore from the basal data furnished by the concentration-time curves for various forms of life is here presented. At first those for some typical mammals will be considered.

The ultimate interest in these curves lies largely in their applicability to human beings. We have little *a priori* knowledge of the principles which underlie the transference of the effects obtained on any particular animal to man. One drug, carbon monoxide, will kill a mouse quickly, where it will only kill a man slowly; another, atropine, will kill a man in doses which have little effect on animals.

In the case of hydrocyanic acid we know little of the laws which govern its absorption by the lung. We know less about the laws which govern its destruction in the body, and least of all about its precise method of attack.

For all these reasons it will be best to start our investigation on broad lines, using animals of very different sizes and very different habits. There is a high degree of probability that, if we know the whole range of toxicity curves for the animal creation, the toxicity of the gas to man will be of the same general order.

## II. THE DETERMINATION OF TOXICITY-CONCENTRATION TIME CURVES.

### (a) *Literature.*

There are no published curves, so far as I know, relating the time of exposure, the concentration of hydrocyanic acid in the air and the prospect of life. Here, let me say, that if I have done less than justice to work carried out in other countries, notably the Central European Powers, it is because during the war, from the very nature of the case, I had no access to their published work.

Some figures of a not very systematic character are given by Henderson and Haggard (1927).

### (b) *Experimental.*

Experiments of the following type were designed to secure more accurate information on this subject.

As a sample experiment four goats were put in an airtight chamber 10 cubic m. in capacity. This chamber was made of sheets of plate glass held together by a steel frame. It contained an electric fan for the purpose of stirring the air, and could be entered from an airlock about 2 cubic m. in capacity.

The operator went in wearing a respirator and taking with him a sealed glass tube which contained the required quantity of hydrocyanic acid; in the present case 3.6 gm. With the door shut the tube was broken in the vicinity of the fan, and the hydrocyanic acid poured out. It evaporated immediately, the air as well as being stirred by the fan was stirred by a large piece of cardboard or paper waved by the operator.

The animals were exposed to this atmosphere which contained "a nominal

concentration of 0.36 mg. of HCN per litre" for 15 minutes (the same figure stands for gm. per cubic metre), after which time the chamber was opened and the animals were withdrawn. Of the four goats, one died and three recovered. The result of the experiment was plotted on a graph in which the ordinate is the concentration of prussic acid, and the abscissa the time of exposure. On such a graph an  $\times$  indicates that the animal died, and an  $\bigcirc$  that it recovered.

By "a nominal concentration" as used above is meant the calculated concentration on the assumption that the material in the tube was pure HCN, that it was all dissipated in the air, and that it remained there. These assumptions are of course "councils of perfection," especially the last, for apart from the possibility of gas being adsorbed on the glass surface, there remains the fact that a considerable amount was inhaled by, and remained in the goats. No effort was made to reinforce the atmosphere in order to compensate for loss. The "nominal concentration" is therefore the "superior limit of concentration."

The order of errors involved in the falling off of the concentration of gas during an experiment may be gathered from the following experiment.

Table I.

Concentration of HCN in air at commencement	Concentration of HCN in air at end	Loss in 38 min. (%)
0.231 mg./litre (1 part in 5250)	0.180 mg./litre (1 part in 6750)	22

Reverting to the experiment under discussion, it was one of a series, the whole results of which are shown in Fig. 1. (Here the concentration of gas is represented in two ways; on the left the molecular concentration, the number of volumes of air which contain 1 volume of prussic acid, according to which the figure includes ranges of between one part of HCN in 1000, and one part in 100,000 of air: on the right-hand side the concentration is expressed in mg./litre.)

Two other experiments were carried out at a concentration of 0.36 mg./litre; at 20 minutes three goats died, and one lived. At 24 minutes all four had died.

Fig. 1 is the result of 18 experiments, with various concentrations of gas, and for various times of exposure. As a result it is possible to draw a curve such that, if a goat is exposed for the specified time to the specified concentration, the chance of its dying will be equal to that of its recovery. This is known as the toxicity curve.

The only points that need be noted here are that the relationship between the "time" and the "dose" is not a simple one (in the case of cumulative poisons it is more nearly hyperbolic), and secondly that the curve at low concentrations becomes parallel to the base line, indicating that there is a limiting concentration of 0.24 mg./litre (1/5000) less than which the average goat could tolerate for an unlimited period.

A general discussion of the theoretical relationship of the factors which

## *Hydrocyanic Acid Gas*

involve the rate at which a gas is absorbed are given by Henderson and Haggard (1927).

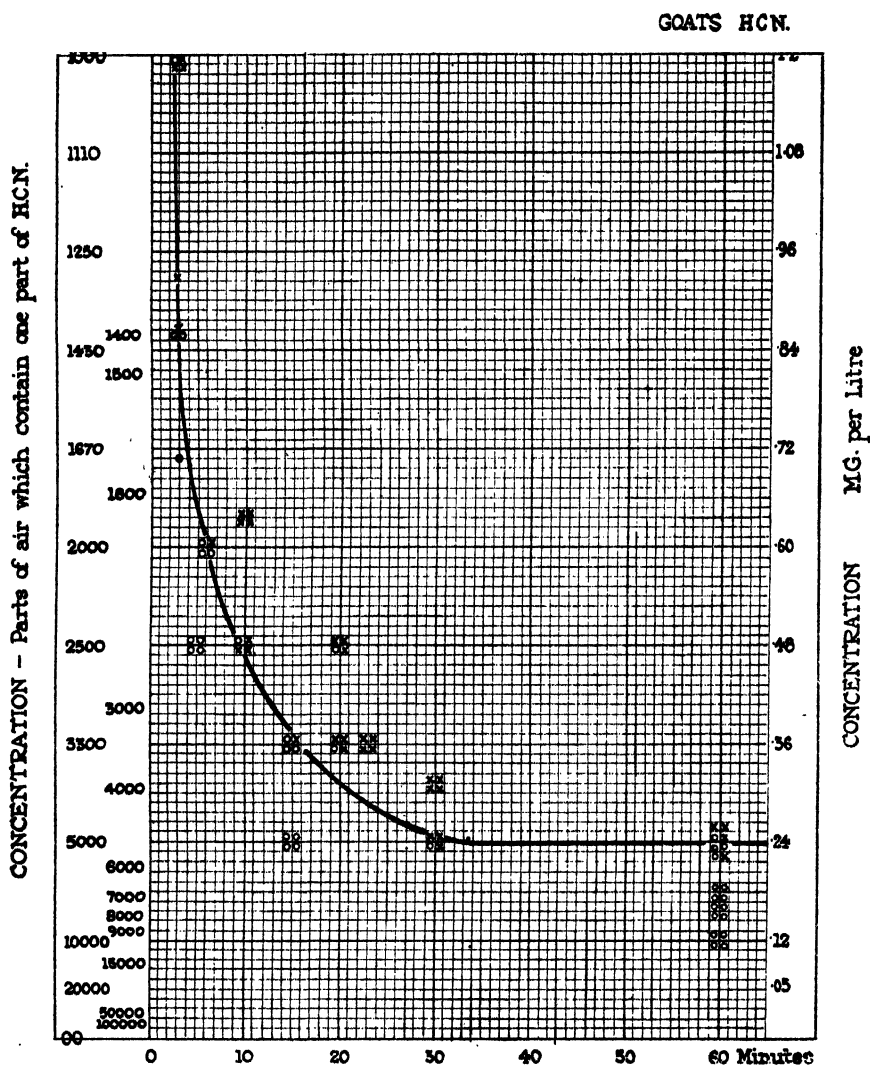


Fig. 1. Shows the prospect of life of goats exposed to atmospheres containing HCN. Ordinate = concentration of HCN in the air. On the left-hand side this is expressed in "molecular" concentration, i.e. the number of parts of air by volume which contain 1 part of HCN. On the right the concentration is given in mg. per litre. Abscissa = minutes of exposure of the animal to the gas. x = the goat died as the result of exposure to the atmosphere. o = the goat survived. The curve, therefore, approximately indicates that for a given concentration and time the goat had a 50 per cent. chance of surviving.

### (c) *Toxicity curves of different mammals and birds.*

Figs. 2-11 show the toxicity curves for monkeys, rabbits, rats, cats, dogs, guinea-pigs, mice, fowls, pigeons and canaries, and except in the case of guinea-pigs, the data on which they are based.

The curves obviously differ greatly; inspection of the actual data on them shows that the difference is far beyond the experimental errors involved.

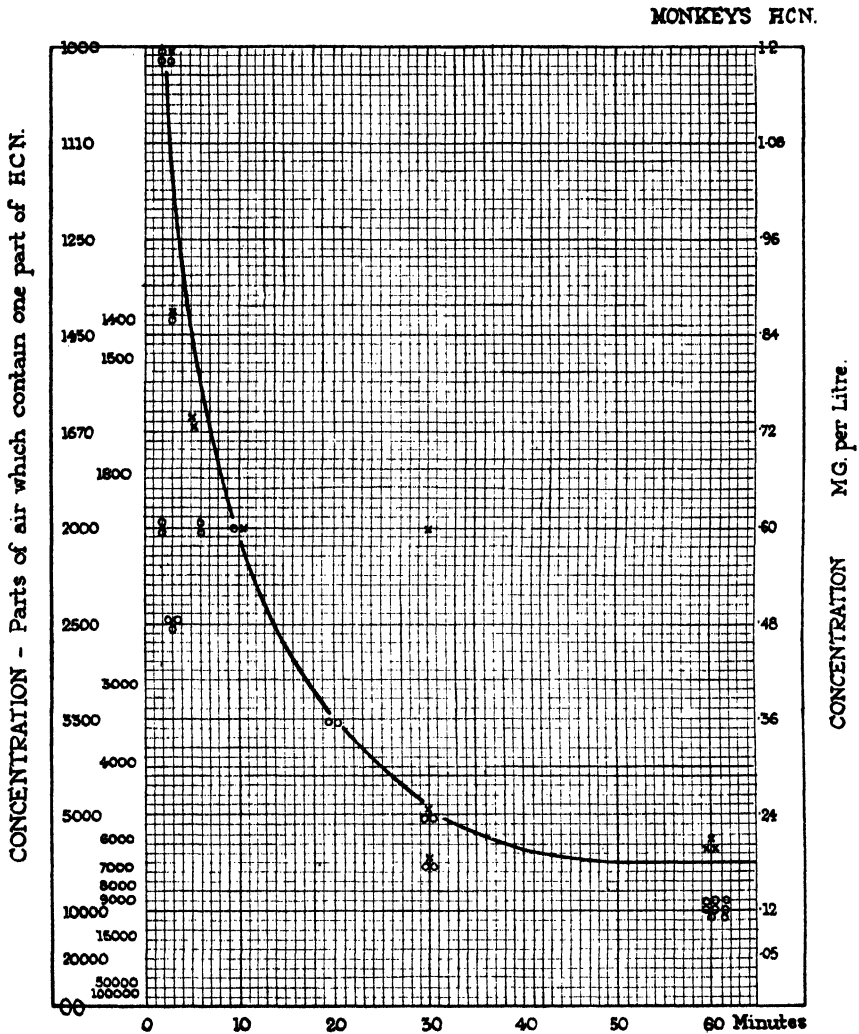


Fig. 2. Shows the prospect of life of monkeys when exposed to atmospheres containing HCN. Scales, etc., as in Fig. 1.

Figs. 12-14 show the individual curves in the preceding experiments superposed. They bring out the fact that there is considerable crossing of the curves.

At the highest concentration about which it is possible to speak with much certainty, about 1.08 mg./litre, the mammals stand in the following order from the most to the least sensitive.

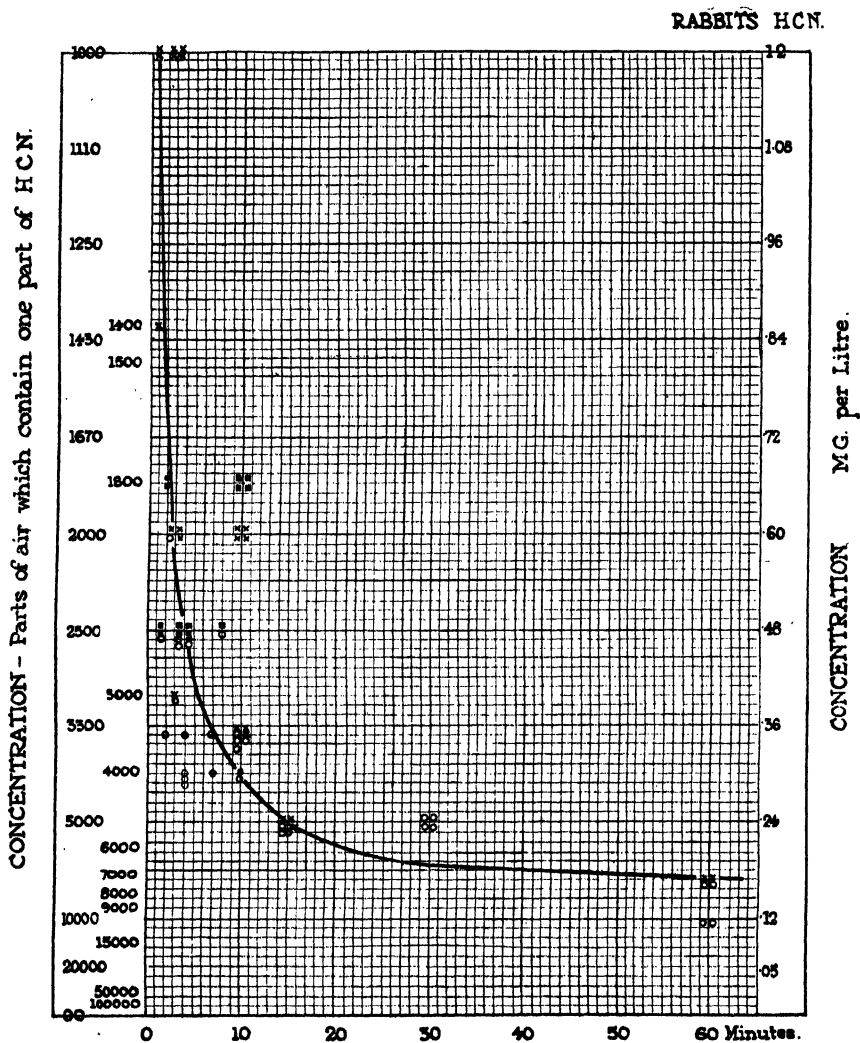
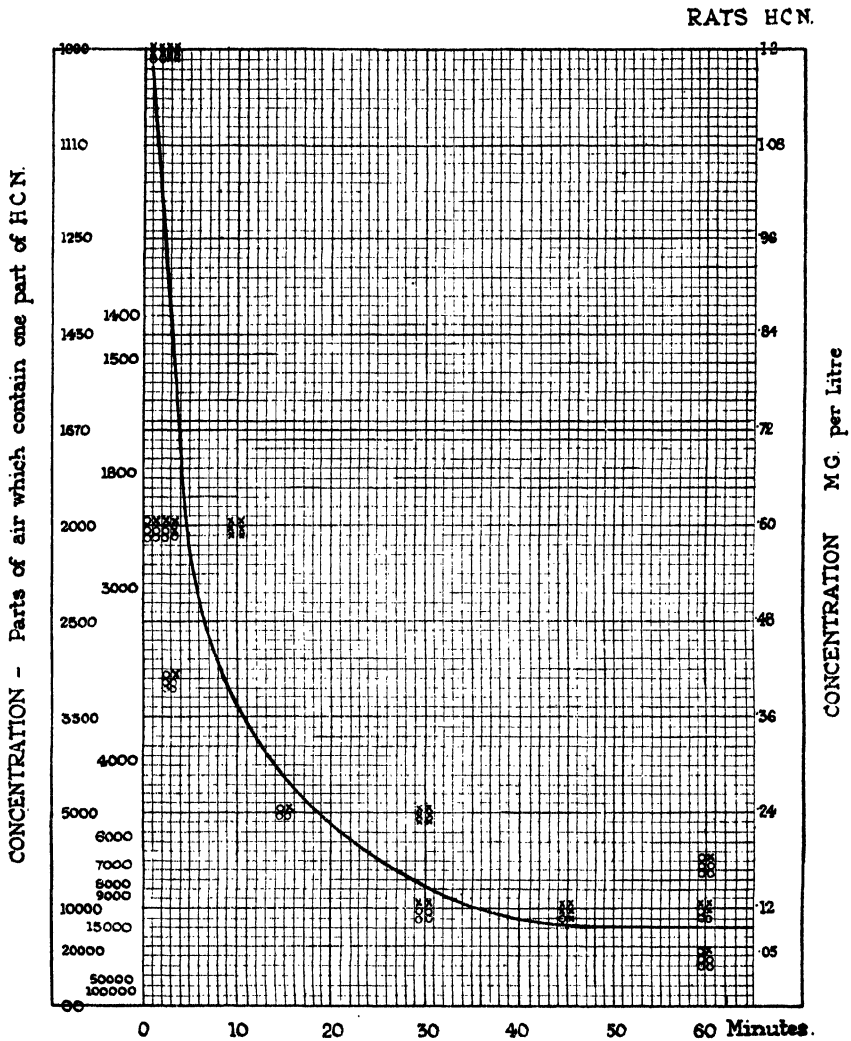


Fig. 3. Shows the prospect of life of rabbits when exposed to atmospheres containing HCN. Scales, etc., as in Fig. 1.





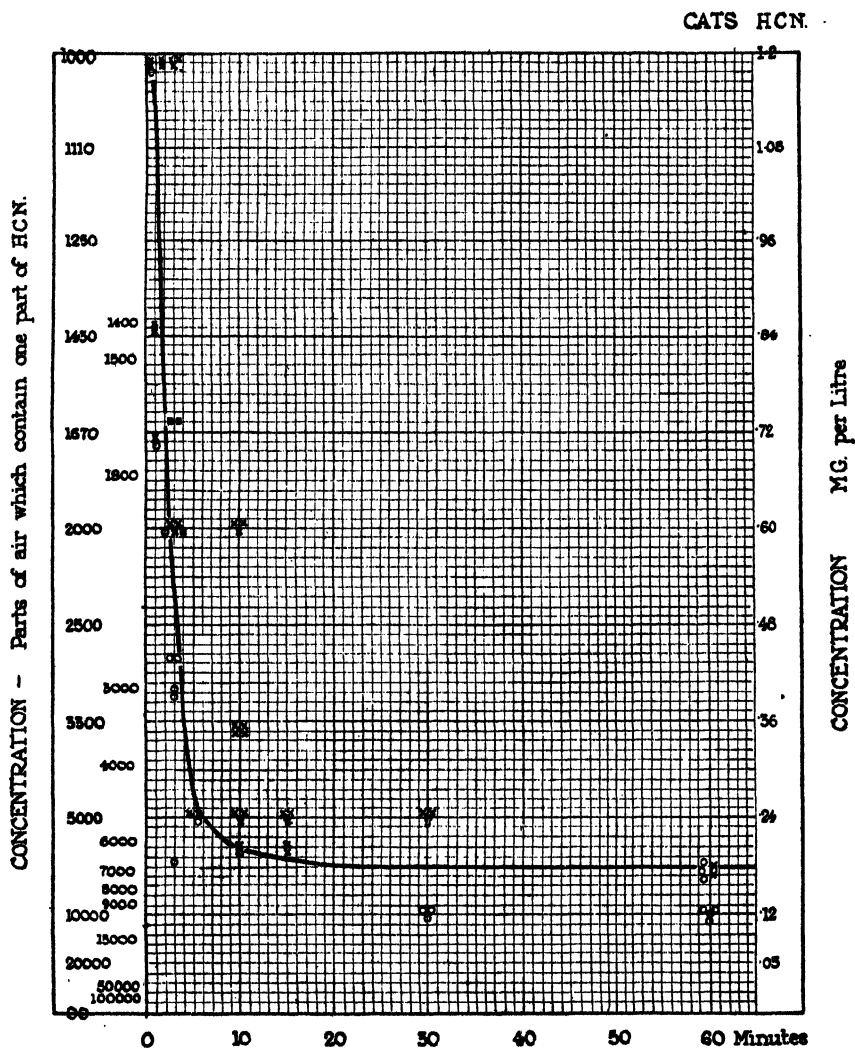


Fig. 5. Shows the prospect of life of cats when exposed to atmospheres containing HCN. Scales, etc., as in Fig. 1.

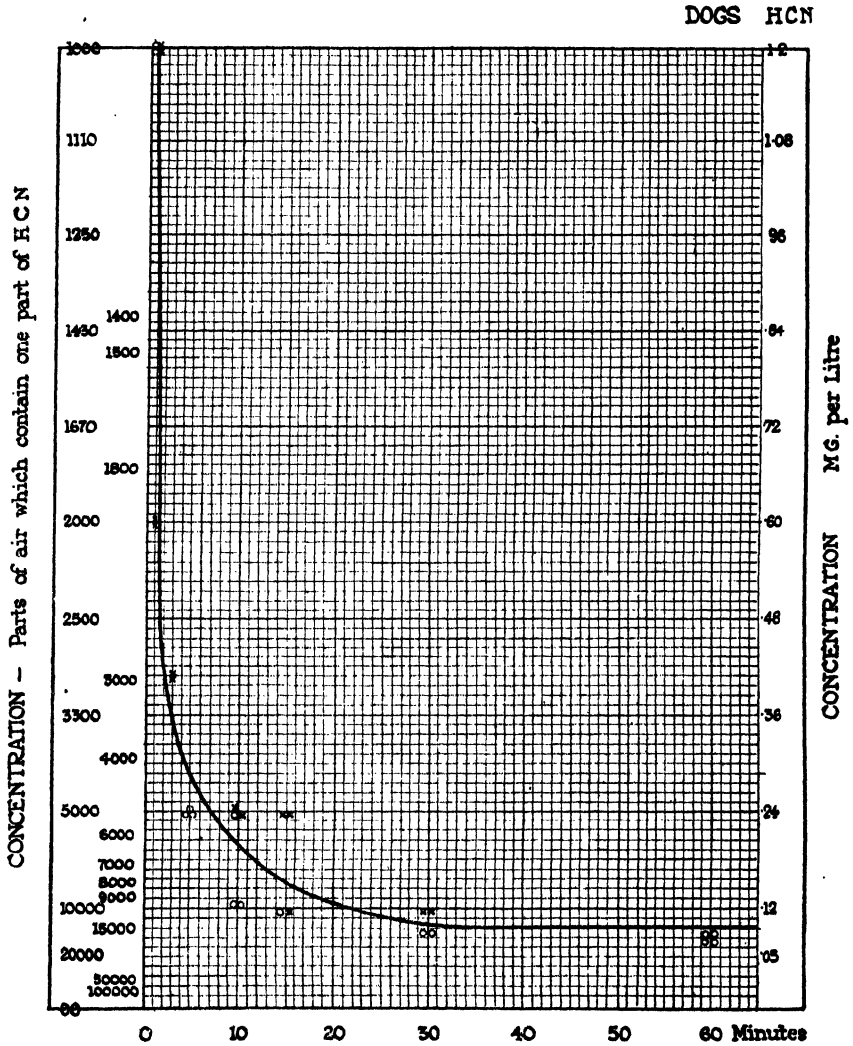


Fig. 6. Shows the prospect of life of dogs when exposed to an atmosphere containing HCN.  
Scales, etc., as in Fig. 1.

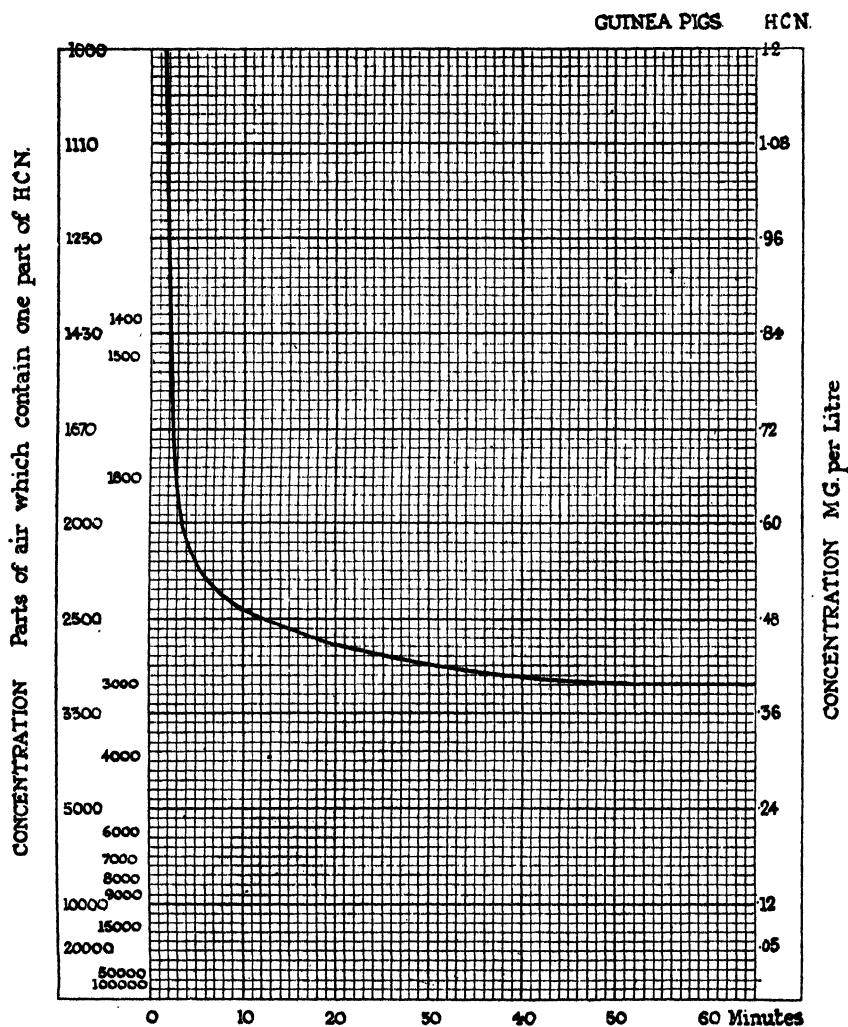


Fig. 7. Shows the prospect of life of guinea-pigs when exposed to an atmosphere of HCN. Scales, etc., as in Fig. 1.

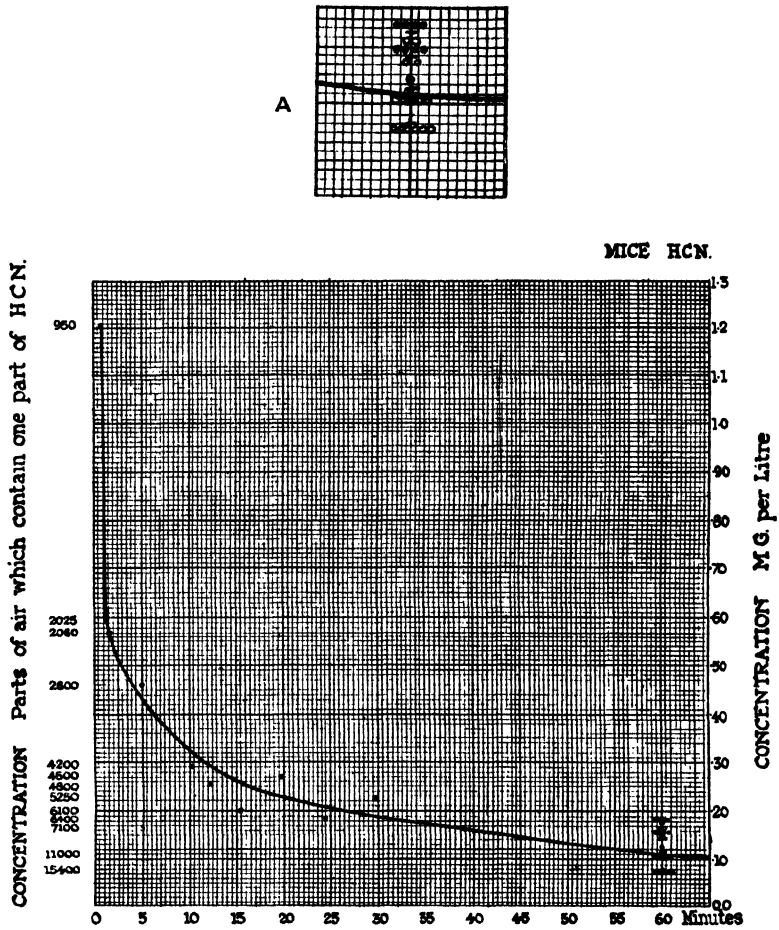
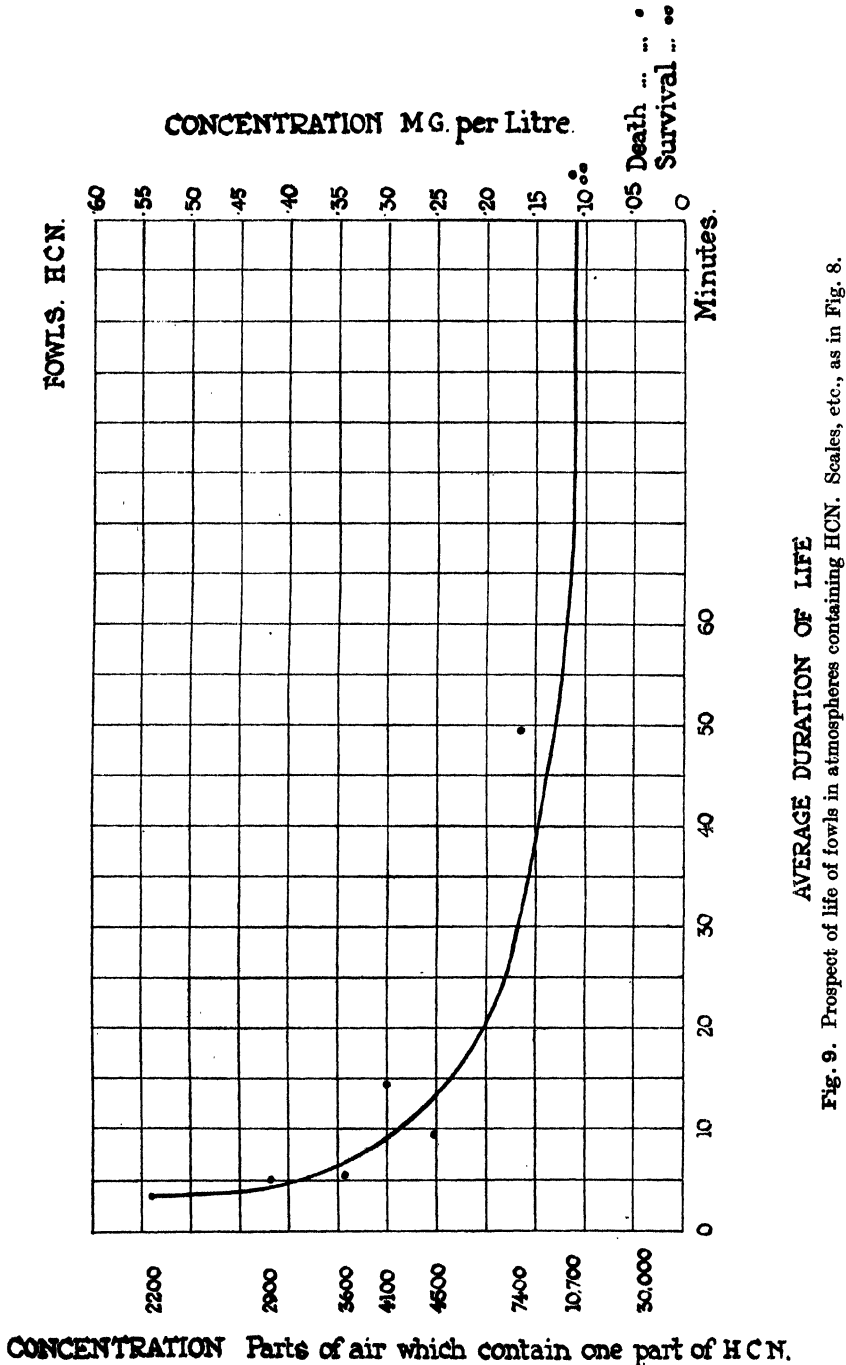


Fig. 8. Shows the prospect of life of mice in an atmosphere of HCN. ● Died. ○ Survived. Scales, etc., as in Fig. 1.

The coarsely grated square A shows details that are obscure in the lower right-hand portion of the figure.



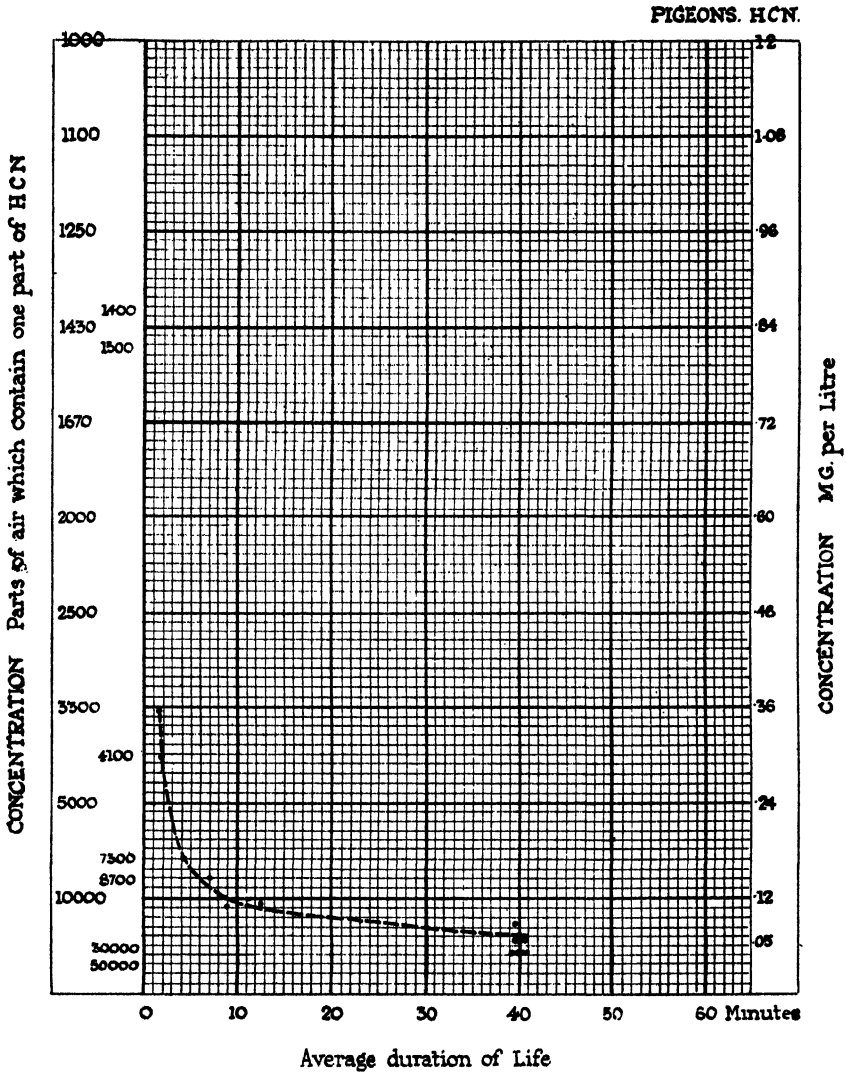
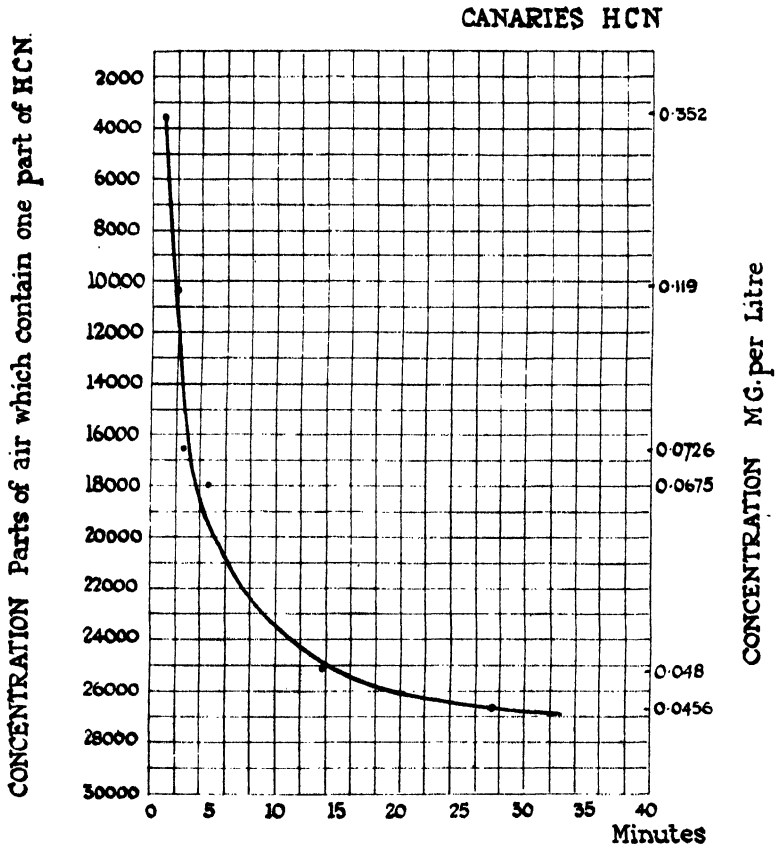


Fig. 10. Prospect of life of pigeons in atmospheres containing HCN. Scales, etc., as in Fig. 8.



**Fig. 11.** Prospect of life of canaries in atmospheres containing HCN. Scales, etc., as in Fig. 8.

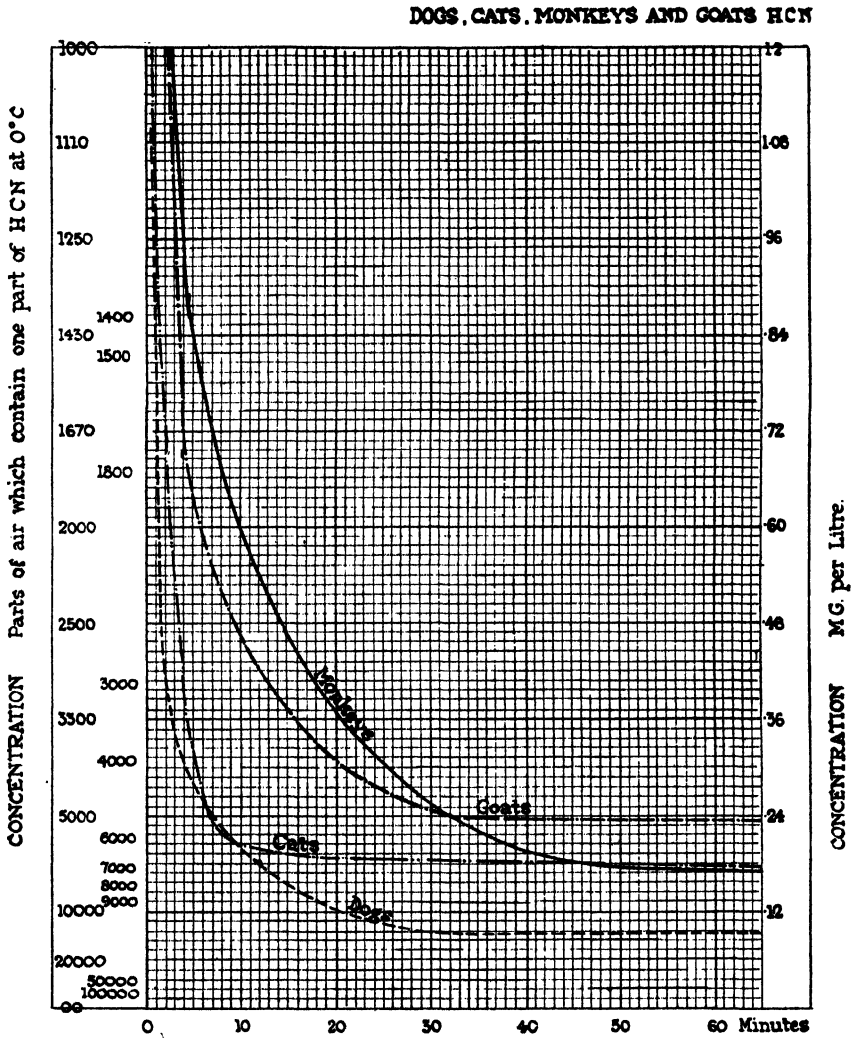


Fig. 12. Shows the curves for the prospect of life given in Figs. 1, 2, 5 and 6, for goats, monkeys, cats and dogs respectively, superposed. Scales, etc., as in Fig. 1.



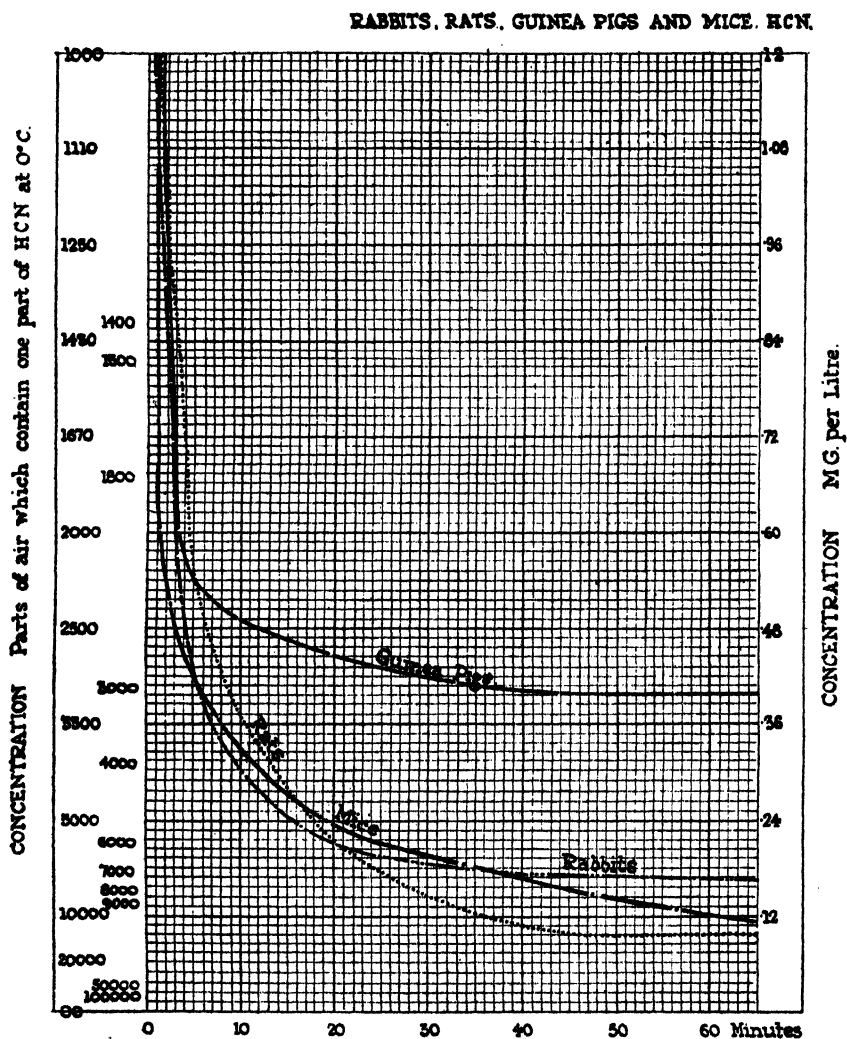


Fig. 13. Shows the curves for the prospect of life given in Figs. 3, 4, 7 and 8, for rabbits, rats, guinea-pigs and mice respectively, superposed. Scales, etc., as in Fig. 1.

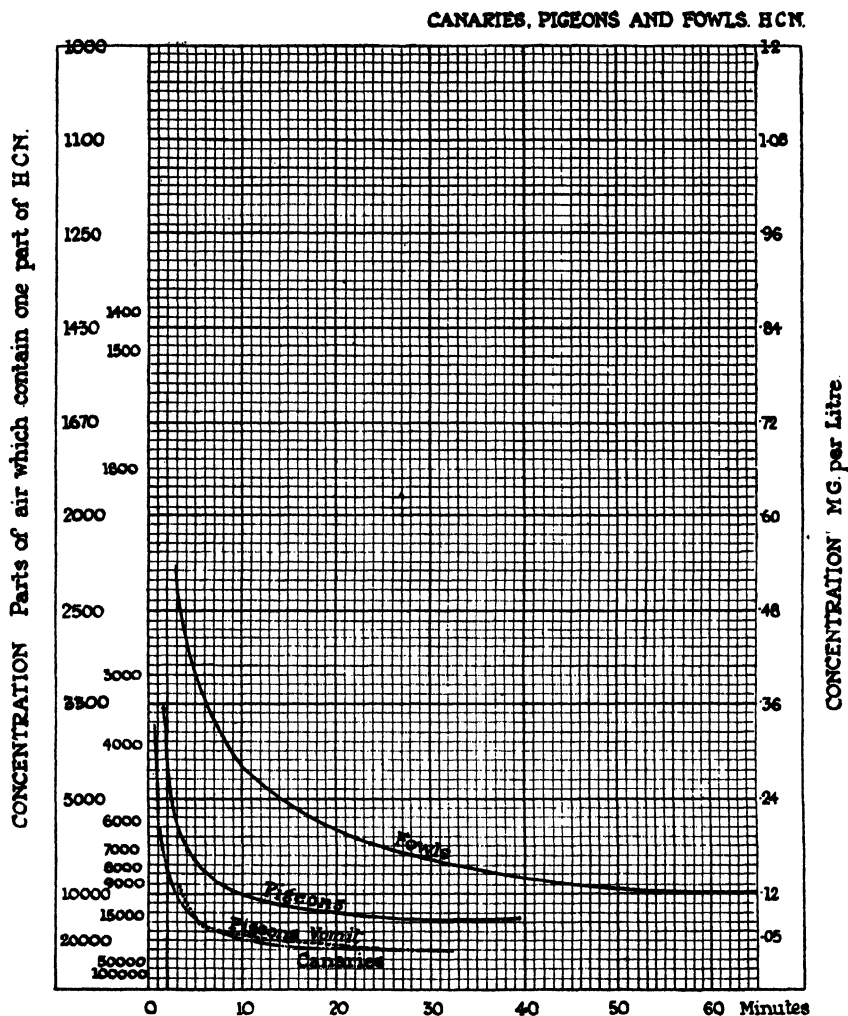


Fig. 14. Shows the curves for the prospect of life, given in Figs. 9, 10 and 11 for fowls, pigeons and canaries respectively, superposed. Scales, etc., as in Fig. 1. Also the times necessary to produce vomiting in pigeons at various concentrations.

Table II. *Animals in order of sensitiveness to HCN.*

Animal	Lethal time of exposure to a concentration of 1.0 mg./litre (mins.)	Animal	Highest approximate concentration which can be breathed indefinitely mg./litre
Dog	0.8	Dog	0.10
Mouse	1.0	Rat	0.10
Cat	1.0	Mouse	0.14
Rabbit	1.0	Rabbit	0.18
Rat	2.0	Monkey	0.18
Guinea-pig	2.0	Cat	0.18
Goat	3.0	Goat	0.24
Monkey	3.5	Guinea-pig	0.40

Table II, the right half, shows also the comparative sensitiveness of the same species as are named in the left half to very low concentrations, the goat being able to tolerate more than twice the concentration which will kill the dog and the guinea-pig four times that which will kill the rat.

The question naturally arises, can any reason be assigned for the difference in susceptibility of different species (1) to weak concentrations; (2) to strong concentrations?

(1) The discussion of weak concentrations is best deferred and is to be found on page 24.

(2) *Strong concentrations.* The case of strong concentrations is in one respect easier than that of weak ones, for it contains one less factor—the rate at which the HCN is destroyed may doubtless be neglected. In the case of carbon monoxide, it is known that if a series of animals of markedly different size are exposed to considerable concentrations of this gas the smaller animals succumb first, because, relatively to their weight, they inhale it and distribute it more rapidly. In a mouse, for instance, carbon monoxide more rapidly attains a lethal concentration in the blood than in a dog, and *a fortiori* than in man. The actual concentration in the blood required to kill the mouse is not smaller than that required to kill a man, in fact it is greater. In very low concentrations, therefore, a mouse might live where a man might die. The present argument, therefore, refers only to concentrations which are in any case lethal, and to the time required in such for a fatality to occur.

Unless there is some reason to the contrary, the whole argument which applies to carbon monoxide might be expected to apply to hydrocyanic acid. The forms of life which pass most air and most blood through their lungs per kg. of animal, per minute, should absorb the gas most rapidly and, other things being equal, succumb soonest. Should the various forms of life not die in the inverse order of their size, when exposed to a given concentration of gas, it can only be, either that their normal rates of respiration and circulation are upset by the gas (a notable instance in point is that of the exposure of rabbits to chloropicrin, the effect of which is to stop their respiration entirely for some minutes), or that the size factor is masked by other and more powerful effects of this gas.

Reference to Table II above shows that the lethal time of exposure to concentrations which kill in a few minutes is not determined primarily by the "size factor." The dog and the goat are not animals of a wholly different order of size, yet the goat will survive more than three times as long as the dog, while the monkey, which again is an animal of about the same size as the dog, will survive four times as long. Yet between the goat and the dog comes a much smaller animal, the rat. The dog and the mouse survive about the same time in high concentrations (0.5–1.0 mg./litre).

The importance of correct ideas on the relation of the "size factor" to the toxicity is very great, for were the size factor the ruling one, it could be taken for granted that man would out-last any of the above forms of life,

and even a likely guess could be made as to the length of time by which he would do so.

The following experiment performed by the Experimental Staff during the Great War illustrates the case of animals of the same order of size, the dog and the goat. The difference in tolerance to HCN of these animals is so great as not to be obscured by the hazards of exposure to gas "in the open." The same difference of sensitiveness between dogs and goats would no doubt be equally apparent in other places even where the gas was distributed somewhat unevenly. The experiment was as follows:

In the open air goats and dogs were exposed to hydrocyanic acid gas in the following way. A number of stations were marked out on the ground; at each station was placed in close juxtaposition a pair of animals, namely one goat and one dog, the animals being selected so that the dog and the goat at any particular station were of much the same size. Commercial hydrocyanic acid gas was then released into the atmosphere from shells, each of which contained about 900 c.c. of the fluid. Since the shells were fired over a short period of time and fell at different distances from the animals, the concentration on the line on which the animals were exposed was somewhat haphazard and uneven. Table III shows the fatality among the dogs and goats respectively. The animals were in no case killed by the shell splinters.

Table III.

	Dogs	Goats
Numbers of animals exposed	7	7
Killed	6	2
Unconscious, but survived	1	0
Affected	7	2

From Table III it is clear that the dogs were much more susceptible than the goats to the HCN vapour.

An experiment in which two dogs and two monkeys were exposed to the same concentration of gas in the gas chamber showed that the monkeys were only commencing to show signs of unsteadiness when the dogs died.

### III. MODE AND SEAT OF ACTION OF HCN VAPOUR.

The effects of hydrocyanic acid, when given by the mouth or injected, are described in all textbooks of pharmacology. In particular the reader is referred to a paper on the subject by Prof. C. Lovatt Evans (1919), the gist of this paper is summarised by Vedder (1925). The general action of HCN in the concentrations under consideration is not, as was formerly supposed, that of functionally destroying the haemoglobin by the formation of cyan-haemoglobin. As Evans showed: "the bright colour of the blood in poisoning is due to the fact that the venous blood contains an excess of oxygen over the normal. In other words the tissues have been unable to remove the oxygen from the blood."

More recently a great volume of work has been carried out by Warburg

(1923, 1928) and his associates, and by Keilin (1925, 1929), showing that the HCN acts on the intercellular enzymes of the body, more especially that known as the cytochrome oxidase. These enzymes are responsible for the oxidative life of the tissues, and are reduced to impotence by prussic acid.

The actual cause of death when small doses of prussic acid are taken is paralysis of the respiratory centre. The acid first stimulates the centre, and then completely inhibits. The heart beat continues after the respiration has ceased, and the heart ultimately fails, partly on account of the direct action of the acid, but partly also on account of the asphyxia secondary to cessation of respiration.

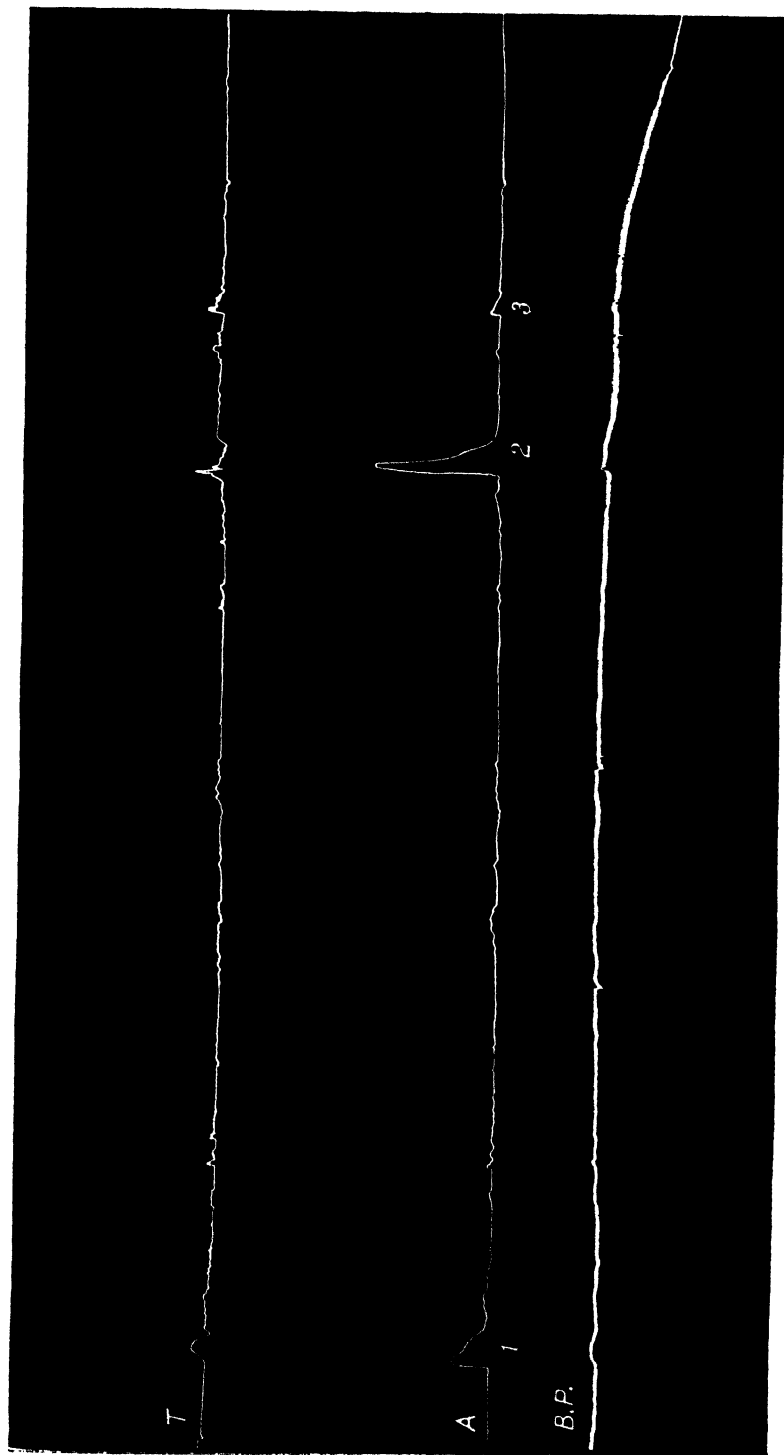
This sequence of events appears to be true also of the prussic acid when inhaled.

Fig. 15 shows three tracings, taken by H. Taylor (1931), *A* and *T* of the movements of the chest of a cat at the moment when life is becoming extinct. The larger oscillations are due to respiration. The third tracing *B.P.* is the arterial pressure, the smaller oscillations on which are evidence of heart beats. The tracings *A* and *T* were taken by Lumsden's method (1923), glycerine tambours having been bandaged to the abdominal and thoracic walls respectively. The alteration in pressure in the tambours were communicated to the recording tambours. The abdominal record gives an indication of the movements of the diaphragm.

What difference can there be between the respiratory centre of, say, a goat and that of a dog which will render the latter more susceptible to hydrocyanic acid than the former? About the dog and the goat in this connection we have no definite information, but experiments performed by H. Taylor on the cat and the rabbit respectively yield instructive results.

The cat, or the rabbit, is lightly anaesthetised, a trachea tube is inserted, and the total ventilation of each may be measured in the following way. The animal is placed in an airtight box (Fig. 16). In the walls of the box are two holes each fitted with a rubber cork. Through the rubber corks pass glass tubes *B'* and *C* respectively. The inner orifice of the glass tube in hole *C* is attached to the trachea tube of the animal; the outer orifice communicates with the toxic atmosphere. The tube *B'* is open inside to the air in the box, whilst its outer orifice is connected by rubber tubing with the orifice *B* of a recording spirometer *A*. Every inspiration of the cat displaces a corresponding quantity of air into the spirometer, which air returns to the box during expiration.

Measurements made in this way of the actual quantity of air inhaled show a remarkable difference between the cat and the rabbit. When HCN is administered in the air the quantity of air which the rabbit inhales per minute is at no stage appreciably increased over and above the value which is obtained when the animal was breathing pure air. In the case of the cat, however, there is at first a great increase in the total ventilation. These facts are illustrated in Figs. 17 and 18, which are records of typical experiments.



**Fig. 15.** Tracings *T* and *A* record movements of thoracic and abdominal walls respectively during the last three respirations 1, 2 and 3, given by a cat.  
*B.P.* = tracing of arterial pressure.

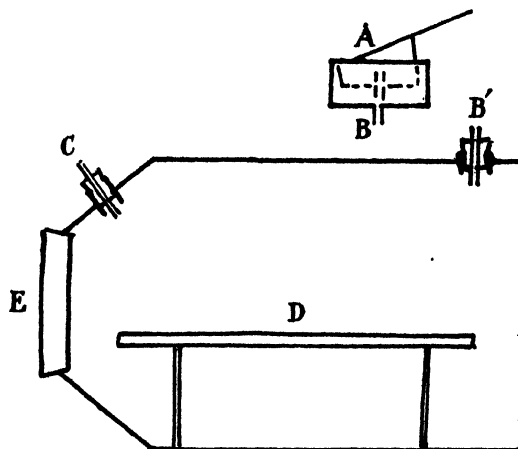


Fig. 16. Apparatus used by H. Taylor for recording the total ventilation and also the rate and depth of respiration. *A*, spirometer. *B* and *B'*, orifices for the attachment of the spirometer to the pneumatic chamber. *C*, orifice leading from the trachea tube to the outer atmosphere. *D*, table to which the anaesthetised animal is attached. *E*, wooden stopper, rendered airtight. The chamber is made from an oil tin. Scale one-fifth of actual linear dimensions.

CAT

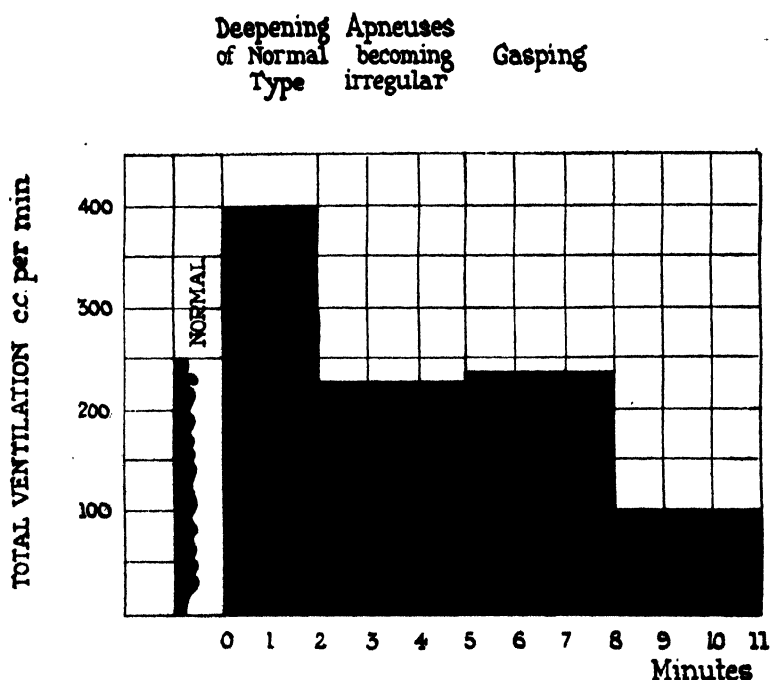


Fig. 17. Variations in the total ventilation of the cat when breathing an atmosphere containing 0.36 gm. of HCN per cubic m. of air. Zero time = commencement of dose.

The essential data in Figs. 16 and 17 may be tabulated as follows:

Table IV.

	Total ventilation before adminis- tration of HCN (c.c.)	Concentration of HCN in air administered in mg./litre	Period from commencement of HCN inhalation till death (min.)	Total volume of air inhaled in that period (c.c.)	Total quantity of HCN inhaled (mg.)
Rabbit	252	0.50	15	1902	0.95
Cat	252	0.36	11	2547	0.92

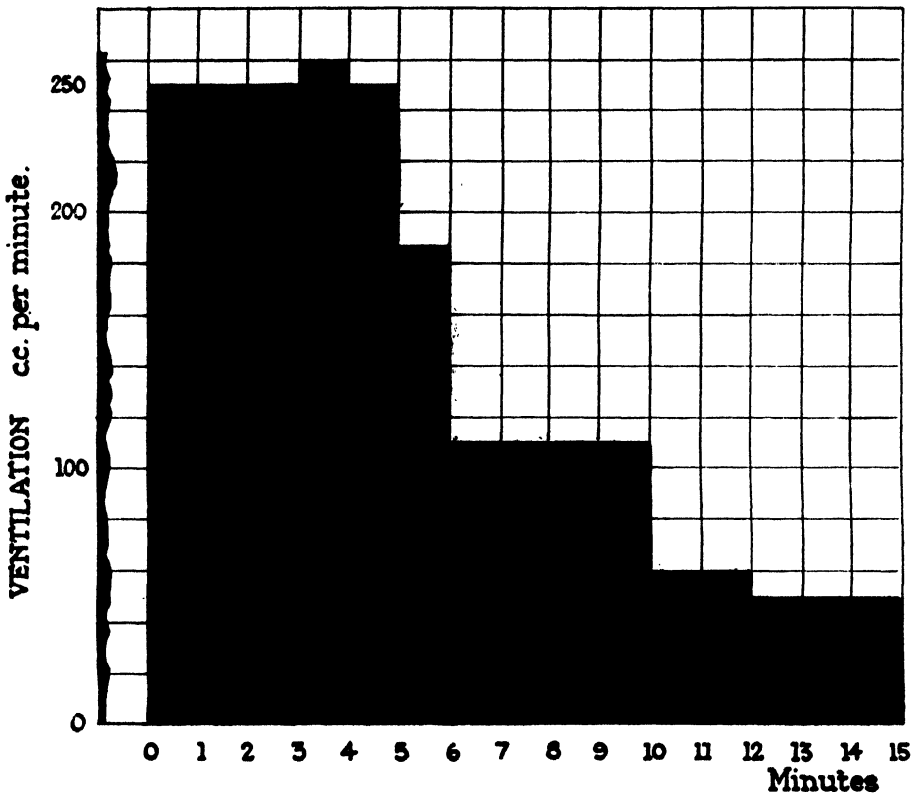
**RABBIT**

Fig. 18. Variations in the total ventilation of the rabbit when breathing an atmosphere of 0.51 gm. of HCN per cubic m. of air.

In spite, therefore, of the facts (1) that the initial ventilation was the same in each case; (2) that the concentration of HCN in the air breathed by the cat was less than in that breathed by the rabbit; and (3) that the cat died sooner than the rabbit, the actual quantity of HCN inhaled by the cat was approximately the same as that inhaled by the rabbit. The lethal dose of NaCN for a cat is given by Evans (1919) as 1.3 mg./kilogram.



At present it is not possible to say that the specific power of hydrocyanic acid to increase the total ventilation is the only factor in determining the specific toxicity of the gas, but clearly it is an important factor.

#### IV. THE SPECIFIC ACTION OF HYDROCYANIC ACID INHALED IN LOW CONCENTRATIONS.

About the factors which dictate the concentrations of hydrocyanic acid which are just tolerable to various forms of life, little can be said. It might be supposed here also that the size of the animal might be the determining factor, the rate at which the gas is inhaled being proportionate to the square of its linear proportions, and the rate at which it is destroyed being proportionate to the mass of the animal. In that case the largest animals would best tolerate the gas. That is not far from being the case, but exceptions exist, and here again the dog forms the principal anomalous case. It must be assumed that one or more of the following factors are at work: (1) that the dog inhales greater quantities of air than would be expected from its size; (2) that its tissues, compared with those of other animals, destroy the gas slowly; (3) that a less concentration in the blood will affect its central respiratory mechanism.

The animal most tolerant of small concentrations of HCN is the guinea-pig.

#### V. THE TOXICITY OF HCN TO MAN WHEN INHALED.

The question of where man stands in relation to the animals is most important. Clearly it is very difficult to obtain exact information on this subject, for when fatalities are concerned the concentrations which produce them are not known.

The experiment about to be described shows, however, that man is not very susceptible. It took place in the airtight chamber already described, the human subject was about 45 years of age, and weighed about 70 kg.

One human being and one dog (about 12 kg.)<sup>1</sup> were exposed simultaneously, without protection, to an atmosphere containing a nominal concentration of 1/1600 hydrocyanic acid, the actual concentration being probably between that and 1/2000. The dog was placed in one corner of the chamber, the man in the corner diagonally opposite to him, so that they faced one another. The utmost care was taken lest, if the dog succumbed first it should not be because its respiration was stimulated by a greater degree of muscular activity, therefore each movement made by the dog was followed by an imitative movement on the part of the man, so that the degree of activity of the two might be as nearly as possible the same. This procedure was followed up to the point at which the dog became unconscious. After that no effort was made by the man to imitate the muscular spasms which form so conspicuous a feature of the later stages of cyanide poisoning. The experiment ran the

<sup>1</sup> The weight of this dog is not recorded and the above figure is guessed from memory of its general appearance. It is intended only to give an idea of the sort of sized dog used.

course as shown in the following notes which were taken by the man as the experiment proceeded.

Table V.

Time from zero	Dog	Man
50 sec.	Became unsteady	—
1 min. 15 sec.	On floor unconscious	—
1 min. 30 sec.	Crying sounds and tetanic convulsions sufficiently established to render it probable that animal was in extremis	—
1 min. 31 sec.	—	Came out of chamber and put on respirator having felt no symptoms. No apparent dyspnoea
1 min. 33 sec.	Respiration apparently* ceased, animal believed to be dead, was pulled out by lead	Re-entered chamber in respirator for purpose of pulling out dog. Having done this he remained outside
5 min.	—	Momentary feeling of nausea
10 min.	—	Attention difficult to concentrate in close conversation

\* Although the corpse was set aside for burial about 6.30 p.m., the dog did in point of fact recover, and was found walking about next morning. It showed no further symptoms.

A report by S. H. Katz and E. S. Longfellow from the American Bureau of Mines issued in July 1923 states: "Men employed in fumigation with HCN have been tested while at rest in 250 parts per million of air for 2 minutes and in 350 parts per million for  $1\frac{1}{2}$  minutes, but felt no dizziness, although possibly on exertion they might have done so. In experiments during the war men have been exposed to 500 parts per million for about a minute without injury<sup>1</sup>. Hydrocyanic acid gas was formerly considered one of the deadliest gases in minute concentrations, but later experience, especially in the war, has shown that man is more resistant than some other forms of life—[Lehmann, *Tabelle der kleinsten Mengen, welche allenfalls ertragen werden*, in book by Kobert, *Kompendium der praktischen Toxikologie zum Gebrauche für Aerzte, Studierende und Medizinalbeamte*. Stuttgart, p. 45] whereas recently Kohn-Abrest [*Notice toxicologique sur les gas*. *Annales des Falsifications*, 8, pp. 215–39] determined that 1000 parts per million are impossible to breathe for many minutes."

## VI. THE CONCENTRATION NECESSARY TO PRODUCE UNCONSCIOUSNESS.

The relation of the time necessary to produce unconsciousness to that necessary to produce death is of much interest, unless either the concentration is dissipated or the man rescued; once he becomes unconscious he must go on breathing the gas and therefore the relation of concentration to time necessary to produce unconsciousness is potentially that which will produce death unless the concentration is sublethal. Such information as is available is chiefly gleaned from experiments on goats. Unconsciousness is, perhaps, the

<sup>1</sup> It is not clear whether the last statement refers to the experiment described above, and which was communicated to the American authorities, or to an independent experiment in America in which the figures were of a similar order.

wrong word, more correctly, the time observed was that at which the animal fell. Table VI gives the data which were obtained during the Great War:

Table VI. *Goats.*

Concentration	Produced unconsciousness in (min.)	Produced death in (min.)
1 in 1000 (1.2 mg./litre)	1	3
1 in 2000 (0.6 mg./litre)	2	10
1 in 3000 (0.4 mg./litre)	6	16
1 in 5000 (0.24 mg./litre)	12	23

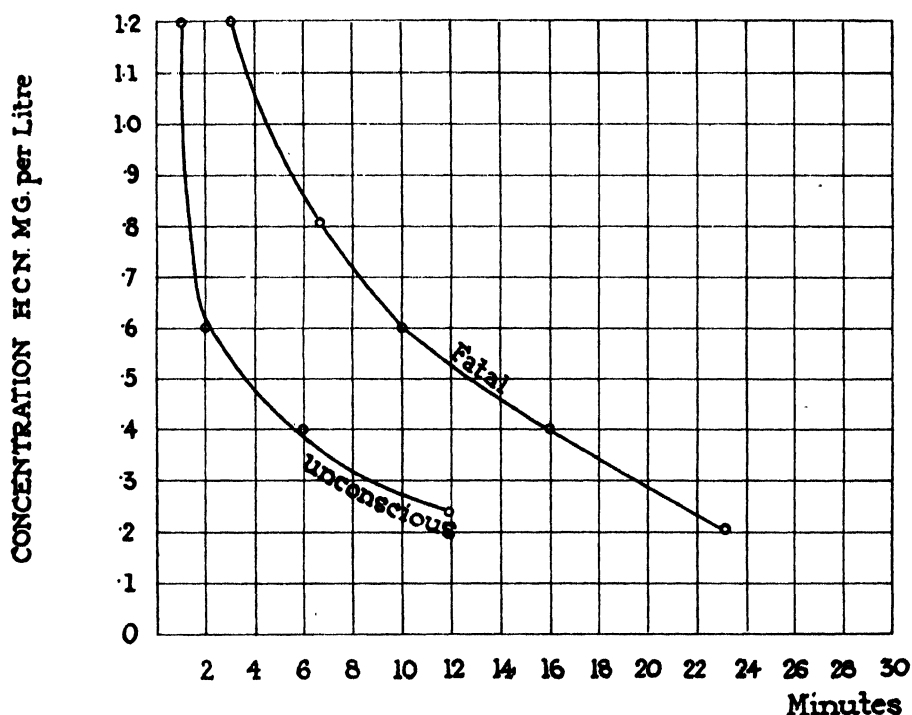
**HCN. GOATS.**

Fig. 19. Curves showing the concentration (ordinate) which if breathed for a given time (abscissa) will produce (a) death, (b) unconsciousness, in 50 per cent. of the cases in goats.

In general, therefore, the time of exposure sufficient for collapse is about one-third of the fatal period of exposure. The rates of the times of exposure necessary to produce collapse and death respectively is not a constant one, and is shown in Fig. 19.

The following figures are quoted from *Noxious Gases* by Henderson and Haggard (1927):

Table VII.

	Parts of air per 1 part of HCN
Slight symptoms after several hours exposure	50,000
Maximum that can be inhaled indefinitely without serious disturbance	20,000-17,000
Dangerous in 30 minutes to 1 hour	7,000- 8,000
Rapidly fatal	3000

## VII. GENERAL COMPARISON BETWEEN MAMMALS AND BIRDS.

Curves have been determined upon canaries, pigeons and fowls. The creatures are of the same order of weight as mice, rats and rabbits respectively. Of the curve for fowls there is nothing special to be said, but one of the most striking things about HCN is its high toxicity for canaries and pigeons. It is about twice as toxic to the former as to the latter. Taking the extreme types, canaries will die in 1 in 10,000 parts of HCN in about 2 minutes; monkeys will withstand that concentration indefinitely. In twice that concentration, 1 in 5000, canaries will die in 1 minute, monkeys in about 25 minutes.

The extreme susceptibility of canaries to HCN makes them valuable as indicators of lethal concentrations of the gas in cases where a chemical indicator is not available. Pigeons, moreover, are no less useful than canaries, for though the concentration lethal to the pigeon is almost twice that lethal to the canary, the pigeon vomits at about the concentration at which the canary dies.

## VIII. THE CONCENTRATION NECESSARY TO MAKE PIGEONS VOMIT.

These may be read from Fig. 14.

Why canaries and pigeons should be so much more susceptible than mice and rats respectively we do not know. Little definite knowledge exists about respiration in birds; it is, of course, of quite a different nature from that of mammals. The air is said to be drawn through the lungs rather than in and out of them, and in this connection we do not know even in mammals what proportion of the HCN in the inspired air is absorbed. One possible explana-

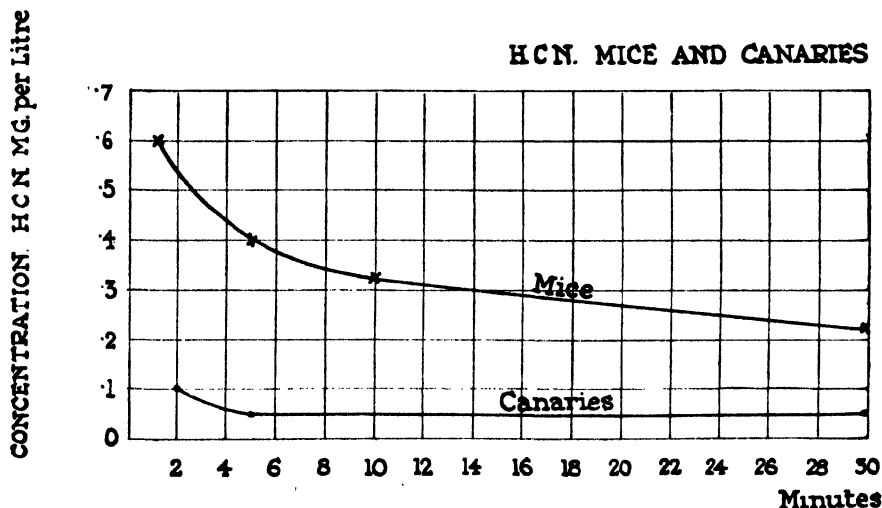


Fig. 20. Comparison of curves relating the fatal concentration to the time for which the gas is inhaled in the case of mice and canaries respectively.

# *Hydrocyanic Acid Gas*

tion has been tested and appears not to be the true one, namely, that birds having a higher metabolism have a much greater total ventilation.

H. Taylor has devised a method for the measurement of the total ventilation which appears to give approximately correct results, it assumes that the

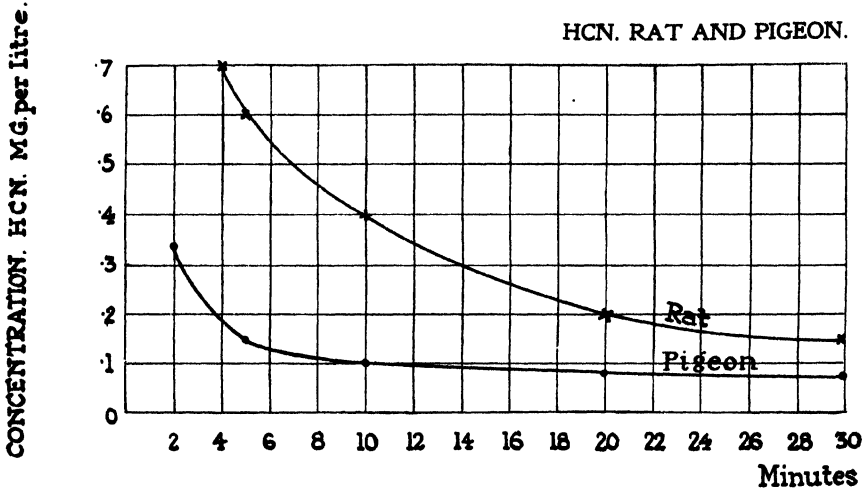


Fig. 21. Comparison of curves relating the fatal concentration to the time for which the gas is inhaled in the case of rats and pigeons respectively.

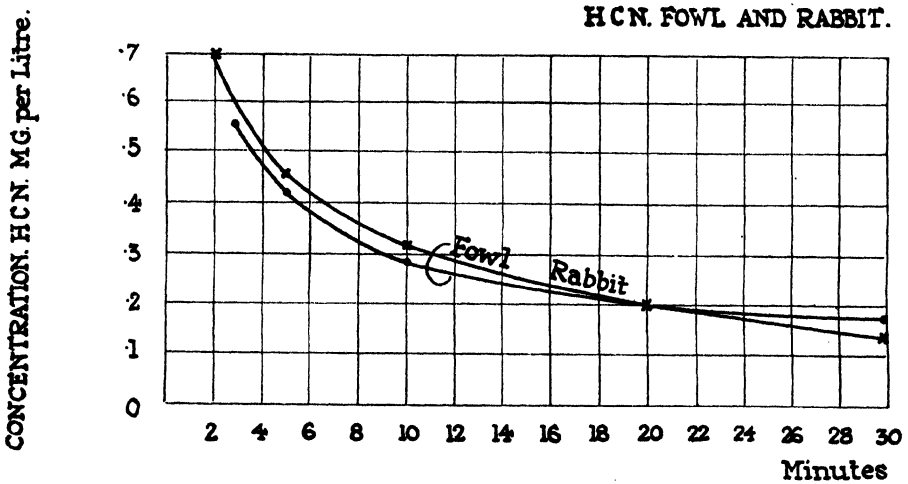


Fig. 22. Comparison of curves relating the fatal concentration to the time for which the gas is inhaled in the case of fowls and rabbits respectively.

expired air is 92 per cent. saturated with water vapour. The animal is placed in a dry chamber, through which a measured current of dry air is passed. The water in the effluent air is collected and estimated, and precautions being taken that the air receives moisture from no other source than the breath,

the volume of the expired air is ascertained. The following data are given by Taylor:

Table VIII.

Animal	No. of experiments	Weight (gm.)	Relation of surface to $W^{\frac{2}{3}}$	Respiration per kg. per min.	Respiration per sq. cm. per min.
Rabbit	15	1256 $\pm$ 184	$\times 12.9$	393 $\pm$ 47	0.33 $\pm$ 0.04
Guinea-pig	27	519 $\pm$ 148	$\times 8.5$	615 $\pm$ 175	0.61 $\pm$ 0.43
Rat	16	205 $\pm$	$\times 9.1$	1561 $\pm$ 273	1.02 $\pm$ 0.18
Pigeon	9	421 $\pm$ 16	$\times 10.45$	1173 $\pm$ 188	0.88 $\pm$ 0.14
Mouse	17	31 $\pm$ 2	$\times 11.4$	5097 $\pm$ 1105	1.41 $\pm$ 0.29
Canary	17	16 $\pm$ 1	10 $\times$ 45	5611 $\pm$ 1099	1.37 $\pm$ 0.27

The rat and the pigeon appear to have much the same total ventilation per sq. cm. of body surface as one another. So also do the mouse and the canary. It is true that the canaries on the average were only half the weight of the mice, but, on the other hand, the pigeons were twice the weight of the rats.

#### IX. INDICATORS.

There is no simple chemical indicator which is affected by hydrocyanic acid which can be trusted to reveal the presence of the gas in dilutions which fall short of those affecting man.

A rather complicated indicator consisting of three sets of test papers, standard sets and tints, a stop-watch, etc. is described by Katz and Longfellow (1923). This professes to detect 1 part in 40,000 of HCN in air.

For the purposes of fumigation it is possible to mix into the prussic acid small quantities of highly irritant gases, which will reveal the presence of the hydrocyanic acid even when the latter is in innocuous concentrations.

The experiments cited above show that many animals, notably dogs, rats, mice, pigeons and canaries, are overcome by this gas before man. In fact, almost any smaller animal is so overcome. Rats and mice may be available, but in their use this caution should be given. A man going into a lethal concentration is taking exercise, at least he is walking, and in addition he may easily be carrying a heavy burden of some kind. Therefore, the margin of safety between the lethal dose for man and that for a mouse or a rat in a cage is by no means so great as might appear from a consideration of the experiment described in Table V.

The dog allows of a safer margin, for in addition to being the most sensitive of the quadrupeds which have been studied, he can be made to walk with the man, and the factor of exercise to that extent eliminated. But undoubtedly by reason of their extreme sensitiveness the best indicators are birds. Two of the most readily available serve excellently for this purpose, the canary and the pigeon. The concentrations at which the pigeon will vomit, or the canary will die, are approximately the same, and are over short periods of time, say up to ten minutes, negligible as compared with the concentration which would affect man.

The subject of the experiment described in Table V was inhaling perhaps

10 litres per minute of gas containing 0.6–0.7 mg./litre of HCN; in 1 minute and 35 seconds he will have inhaled say 10 mg. of the gas. To inhale the same quantity of HCN (which is known to be harmless) the man under the circumstances of the experiment would require 10 minutes if the concentration were 0.1 mg. per litre. In this concentration the canary would be dead in about 2 minutes, in which time the pigeon also would be vomiting.

Moreover, the man has in his favour the extra margin obtained by the oxidation of the gas in his tissues, so that the content of HCN in his blood would be by no means so high if he inhaled 1 mg. of HCN in  $1\frac{1}{2}$  minutes as if he took 10 minutes for its inhalation. On the other hand, if the man was so far exerting himself as to be inhaling 50 litres of air per minute (the maximum possible to most men being of the order of 100 litres per minute), the picture would alter, and the value of the bird as an indicator would be reduced.

It should, therefore, be emphasised that persons entering suspect atmospheres should not indulge in greater exertion than is necessary. They should not be breathing heavily.

#### X. TREATMENT.

The possibilities of treatment are:

(1) Artificial respiration.

(2) The administration of materials calculated to “kill” the HCN in the body. These may be considered either (a) as prophylactic measures, (b) as remedial measures.

##### (a) *Efficacy of artificial respiration.*

Artificial respiration is certainly useful, and should be employed.

Whilst permanent lesions of the brain are believed to have been found in certain cases, the general trend of knowledge with regard to HCN poisoning is like that of ether or alcohol; the effect is reversible, and if life can be maintained until the lethal concentration has been dispelled the machinery of the body is not impaired, it is merely temporarily brought to a standstill.

The phrase used above “if life can be maintained” is advisedly a loose one. Closer enquiry shows that the impairment of the vital process consists in an incapacity on the part of the tissue ferments, responsible for the fundamental oxidation processes of life, to take up oxygen and build the oxygen into living material. The essential spot at which this disability spells fatality is the living material of the cells in the brain which performs the function of respiration. This blocking of the oxidative enzyme machinery is not diminished by the accumulation of oxygen, and therefore no benefit on this count can be hoped for, from artificial respiration. On the other hand, if all the other mechanisms of the body can be preserved unimpaired till the oxidative enzymes are freed from HCN, the functions of the body will become re-established. The fate of the animal is staked upon a race between the onset of incidental degradation of the machinery of the body, and the dissipation

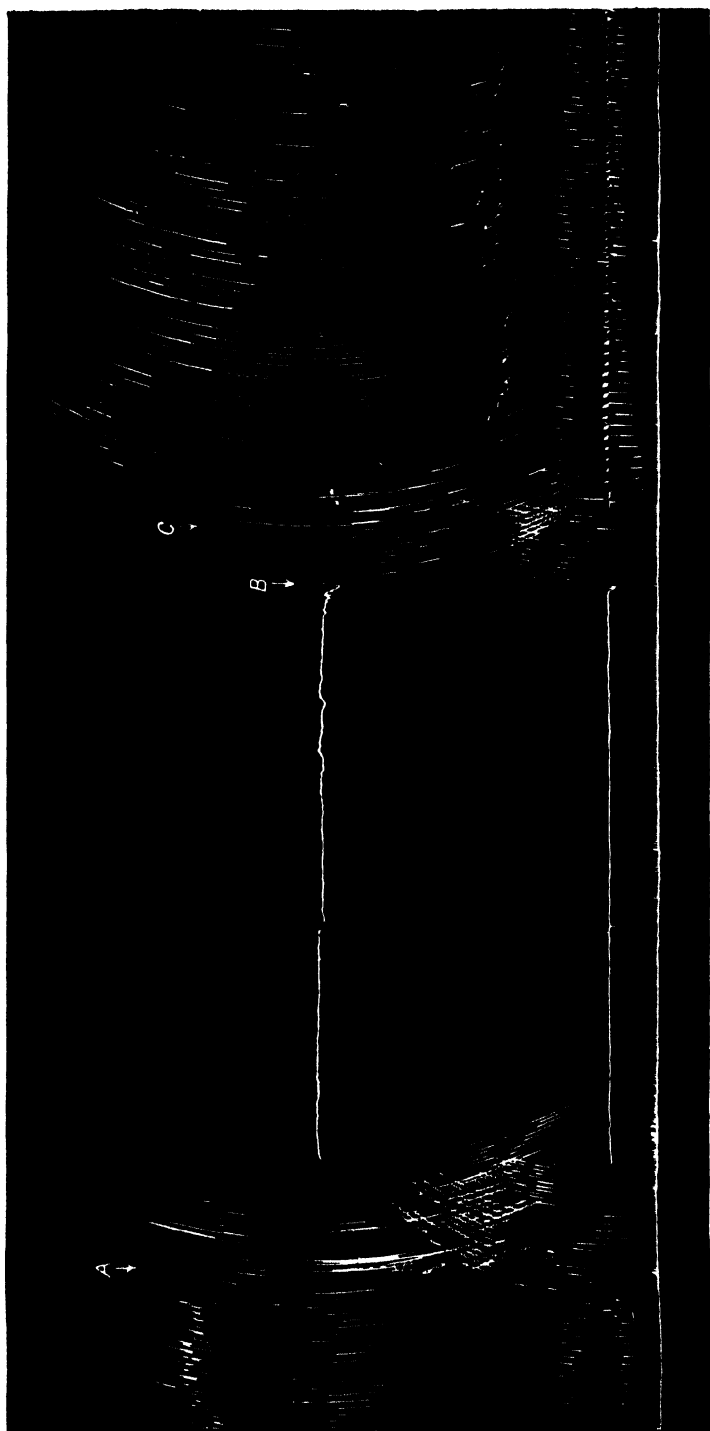


Fig. 23. Record of respiration of the rabbit. Time = seconds (for explanation, see text p. 32).



or "destruction" of HCN. The terms ( $\alpha$ ) "dissipation," and ( $\beta$ ) "destruction," suggests the two lines along which artificial respiration may be useful.

( $\alpha$ ) *Dissipation.* During artificial respiration HCN imparts its odour to the expired air, from which it may be inferred that the concentration of the gas in the blood is reduced, HCN being given off in the air.

( $\beta$ ) *Destruction.* Hydrocyanic acid is destroyed in the tissues. This destruction is probably a change to sulphocyanide and oxidation, yet it does not apparently depend upon the type of enzyme action which HCN destroys. Therefore, if HCN and oxygen can be kept circulating in the body the HCN will be gradually destroyed, and the velocity of this destruction may depend upon the concentration of the oxygen present, following the general law of mass action. Therefore, while no quite definite statement can be made, it seems not unlikely that artificial respiration with oxygen is more desirable than artificial respiration with air.

The upper tracing shown in Fig. 23 is that of a lead attached to a strip of diaphragm muscle in the rabbit (Head's method), and therefore registers the movement of one of the respiratory muscles. The lower tracing shows one of alteration of pressure in the trachea. When the animal inspires this tracing rises, but when on the other hand the lung is artificially inflated, the tracing falls. Reading from left to right, at the commencement of the tracing, the animal was breathing an atmosphere containing HCN at the dictate of its respiratory centre. At *A* artificial respiration was commenced, and the lung was inflated with the same atmosphere, but the total ventilation was considerably increased with the result that the rabbit ceased breathing, the artificial respiration was stopped, and had no further steps been taken the animal would probably not have again respired; but at *B* resort was had to artificial respiration of cyanide-free air; the respiratory centre woke up at *C*, and the animal was restored; this procedure was repeated several times on the animal in question.

(*b*) *Administration of glucose.*

Violle (1926) and Forst (1928) have advocated the injection of glucose on the ground that HCN will react with the glucose forming cyanohydrin, which undergoes hydrolysis to form a harmless compound. The merits of glucose have been denied by Hynd (1927) and Heymans and Soenen (1927). Taylor (1930) has repeated both the experiments of Forst and those of Violle.

Forst's method consisted in the injection of glucose previously to the injection of cyanide. As an example, a rabbit of 2.6 kg. weight was given an injection of 6 c.c. of glucose solution (50 per cent. glucose in 0.85 per cent. saline). Eighteen minutes later 2.1 c.cm. of 0.12 per cent. HCN (*i.e.* 0.97 mg./kg.) were injected. Taylor's experiments showed that glucose had a certain value in marginal cases.

	Died	Recovered	Total
Following Forst's procedure with glucose	5	4	9
Controls without glucose	7	2	9
With twice the dose of glucose	3	6	9
Controls without glucose	8	1	9

The following experiment is an example of Violle's procedure:

40 c.c. of 10 per cent. glucose were injected intravenously, and 60 c.c. into the peritoneal cavity. One hour afterwards the animal was exposed to air containing HCN 1.79 gm. per cubic m. Twelve pairs of rabbits were subjected to the above procedure (with, in some cases, slight variations) the glucose gave no immunity to the HCN, when the latter was inhaled.

As a prophylactic against HCN vapour glucose, therefore, has no practical value, and therefore from the practical point of view the subject may be dismissed. On the theoretical side two or three interesting points arise.

(1) Why should glucose reduce the susceptibility of the animal to HCN when the latter is injected, but not when it is inhaled? The probable answer is that in the case of injection, once the dose is introduced, the body has no further access to HCN, but in the case of inhalation there is always a background of HCN which at each circuit of the blood through the lungs, replenishes what has been oxidised, or at least does so as a first approximation.

If the explanation is correct, glucose should benefit a case which after a short period of inhalation of HCN was taken out of the toxic atmosphere.

(2) Does the glucose act in reality chemically? There is a possible alternative to this idea that the glucose acts by transforming the cyanide into cyanohydrin. It is that the glucose increases (as indeed it does) the volume of the blood, and that the same injection put into a greater volume of blood produces a less concentration of cyanide in the blood, and consequently a blood stream less toxic to the tissues. This simple explanation seems not to be the correct one, for according to Taylor's researches, cane sugar, which produces similar osmotic effects in the body, does not increase the tolerance of the body to HCN. Moreover, dilution of the blood with "gelatine saline" does not increase the tolerance. On the other hand, acetone, which acts chemically like glucose, produces a similar degree of resistance to HCN on the part of the animal.

*Rabbits.* 0.4 c.c. per kg. of 26 per cent. acetone injected subsequently given 1.1 mg. HCN per kg. subcutaneously.

	Died	Recovered	Total
Acetone animals	2	6	8
Control animals	5	3	8

The benefit is, however, only in the marginal cases administration of 1.2 mg./kg. of HCN killed all the animals, whether given acetone or not.

### (c) *Administration of Nitrites.*

The most recent suggestion made by Mlodveanu and Gheorghin (1929) is the use of sodium nitrite as an antidote against HCN. The suggestion is that the nitrite reacts with urea in the body, produces CO<sub>2</sub> and so stimulates the respiratory centre. It is claimed that after a dog has ceased breathing as the result of HCN, respiration may be started again on the injection of sodium

nitrite. "If several minutes after the stoppage of respiration an intravenous injection of 10–20 c.c. of a freshly prepared 1 per cent. solution of nitrite be injected" respiration will be restored. These experiments have as yet been incompletely controlled.

## REFERENCES.

- EVANS, C. LOVATT (1919). *J. Physiol.* **53**, 17.  
FORST (1928). *Arch. f. exp. Path. u. Pharm.* **128**, 1.  
HENDERSON and HAGGARD (1927). *Noxious Gases*, p. 110. New York.  
HEYMANS and SOENEN (1927). *C.R. Soc. Biol.* **96**, 202.  
HYND (1927). *Biochem. J.* **21**, 1904.  
KATZ and LONGFELLOW (1923). *Serial Report No. 2405 of the American Bureau of Mines*.  
KEILIN, D. (1925). *Proc. Roy. Soc. B*, **98**, 312.  
—— (1929). *Ibid.* **B**, **104**, 206.  
LUMSDEN (1923). *J. Physiol.* **57**, 357.  
MLODVEANU and GHEORGHIU (1929). *C.R. Soc. Biol.* **102**, 164.  
TAYLOR, H. (1930). *J. Physiol.* **69**, 124.  
—— (1931). *Ibid.* (in press).  
VEDDER (1925). *The Medical Aspects of Chemical Warfare*. Baltimore.  
VIOLE (1926). *Bull. Acad. méd.* **95**, 664.  
WARBURG, O. (1923). *Biochem. Zeitschr.* **136**, 266.  
—— (1928). *Ueber die katalytische Wirkung der lebendigen Substanz*. Berlin.

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# NATURAL BACTERICIDAL ANTIBODIES: OBSERVATIONS ON THE BACTERICIDAL MECHANISM OF NORMAL SERUM.

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## INTRODUCTION.

THE natural occurrence of antibody-like principles in the serum of various animals has been the subject of recent systematic studies by Mackie and Watson (1926), Mackie and Finkelstein (1928, 1930) and Gibson (1930). An important result of this work has been the demonstration in normal sera of specific complement-fixing and agglutinating agents for a wide variety of bacterial antigens. Different animal species vary, however, as regards the occurrence and degree of activity of these principles. Bacteria vary also in their reactivity. Serological analyses have established the antibody-like character of the serum principles responsible for these reactions, though they undoubtedly differ in certain respects from the antibodies produced by immunisation.

The data collected in this way regarding the occurrence and characteristics of "natural" antibodies are of interest from a general biological standpoint, and have an important bearing on immunological theory. These questions have been discussed in the publications cited above.

As a corollary to such studies and in the light of their results, a systematic inquiry has been instituted into the bactericidal action of normal serum. The immediate object in view has been to analyse the mechanism of this phenomenon, with particular reference to the part played by antibody-like principles, and the characters and behaviour of these substances.

Since the classical observations of Nuttall (1888), Buchner (1889) and others, first contributed to our knowledge of the subject, it has been recognised that this property of serum is a variable one, differing according to the animal species and the type of bacterium, but, except in certain particular instances, the variations have not been fully defined and interpreted from the biological and immunological standpoints. There has also been considerable uncertainty regarding the mechanisms involved in the bactericidal property of normal serum, though the factors concerned in the same effect produced by a specific immune serum have been more clearly defined.

The thermolability of the normal bactericidin of blood serum towards certain bacteria was demonstrated by the early workers on the subject, and later work on immune cytolytic sera has identified the thermolabile bactericidal principle in normal serum with "complement" as defined by Ehrlich. On this basis, the mechanism of normal bactericidal action towards certain

bacteria was carefully studied by Muir and Browning (1908). They concluded that this reaction may, in certain cases, differ from the same effect produced by an immune serum, but they left the question open whether the effect is mediated by a natural sensitising immune body or is due to the direct action of a "bacteriophilic" complement. It has been generally assumed, however, that the reaction is analogous to that of an immune serum and that a sensitising antibody plays a part in the phenomenon, though the possibility of bactericidal action by complement alone has not been definitely excluded. While the existence of certain natural bactericidal antibodies has been proved, there has been no clear evidence to show that the corresponding action of normal serum generally is dependent on such principles.

There has also been uncertainty regarding the specificity or non-specificity of natural bactericidal effects. Muir and Browning (1908) reviewed the literature on this question prior to their own work, and studied the specificity of these reactions by absorption methods. They found that treatment of a normal serum with increasing amounts of bacterial emulsion produced first a diminution of the bactericidal action towards the homologous bacterium, and then a decrease in the effect of the serum on other bacteria. The studies of natural complement-fixing and agglutinating antibodies (referred to above) have suggested the likelihood that the bactericidal effects of normal serum may be due to multiple specific antibodies sensitising bacteria to the lytic action of complement. Recently Gordon and Wormall (1928) have shown how bacteriolysis of *B. dysenteriae* (Flexner) by normal guinea-pig serum depends on the combined action of complement and a thermostable factor removed from the serum by absorption with the particular organism.

The question is further complicated by the fact that different mechanisms may be concerned in the bactericidal action of normal sera, and that the factors may vary with different organisms. The killing of anthrax bacilli by normal serum was attributed by Gruber and Futaki (1907) to a product of blood platelets ("plakanthrakocidine") which is independent of complement. Leucocytic extracts ("leukins") have been shown to possess marked bactericidal properties for certain bacteria (see Ledingham, 1922). Pettersson (1926, 1927-8) has classified the bactericidal agents of serum into  $\alpha$ -lysins and  $\beta$ -lysins. The former apparently represents the complement acting along with a sensitising agent analogous to an immune body. The latter, according to Pettersson, consists of a stable "activating" agent (resisting a temperature of 63° C. for half an hour) and an "activable" principle which unites with the bacteria in the presence of the activating agent. He differentiates the organisms that are susceptible to the  $\alpha$ - and  $\beta$ -lysins respectively. The occurrence of heat-stable bactericidal properties in serum has also been studied by Selter (1918), who inclined to the view that different organisms are affected by a common agent and not by multiple specific substances. It is noteworthy that Seiffert (1912, 1917) regarded the bactericidal properties of a serum as due to specific agents.

The whole subject of the bactericidal action of normal serum has been reviewed recently by Knorr (1929).

In the studies recorded in this paper attention has been directed mainly to those bactericidal properties which have proved labile at 55° C. and dependent, therefore, on the action of a labile complement-like agent. The essential questions at issue have been whether these effects are mediated by antibodies and the properties and reactions of such substances.

### METHODS.

The following methods of estimating bactericidal action have been devised for the special purpose of carrying out large numbers of quantitative and comparative tests with the maximum facility compatible with accuracy and reliability, and were used in this investigation.

#### METHOD I.

In testing organisms which did not rapidly lose viability when suspended in salt solution, a given quantity of serum was mixed with a given volume of varying dilutions of bacteria made up in 0.85 per cent. sodium chloride solution; after incubation at 37° C. the relative sterilising action of the serum was determined by cultivating a standard loopful of each mixture, a stroke inoculation being made on a plate of the appropriate medium. A parallel control test was carried out by cultivating similarly loopfuls of the varying bacterial dilutions, to the given volume of which saline solution had been added instead of serum, these suspensions having been incubated previously along with the test series. The degree of bactericidal effect could in this way be determined by comparison of the end-points of growth in the two series, growth being absent from concentrations in the test series which yielded growth in the control (see Table I). Lack of bactericidal action was indicated by coincidence of the two end-points (see Table II). The bacterial dilutions were prepared from 18–24 hours' cultures.

This method gave satisfactory results with certain organisms, *e.g.* Staphylococci, the *coli*-typhoid and allied groups, *V. cholerae*, *B. pyocyaneus*, *B. proteus*, etc., which maintained their viability in saline solution during short periods of incubation (*e.g.* 3 hours) so that no appreciable alteration in the number of viable organisms in the bacterial dilutions occurred during the course of the test (see Tables III and IV). It had the advantage that the comparative bactericidal power of a large number of specimens of serum towards a particular organism could be estimated by one set of loop-transfers made at the end of the period of incubation (cp. Method II *infra*).

The mixtures were all carefully shaken before making transfers to ensure uniformity of the bacterial suspensions. The possibility of agglutination of the suspended organisms influencing results and introducing a fallacy was considered but did not appear to affect the validity of these tests; for example, certain organisms were strongly agglutinated by various sera (evidenced by visible clumping when serum was added to the standard suspension) without the least sign of bactericidal effect as judged by the results of the test.

The comparison of end-points was also checked by estimates of the approximate amount of growth resulting from each transfer. The relative sterility of the serum was controlled by a loop-transfer from the given quantity of serum mixed with an amount of saline equal to that of the bacterial dilutions and incubated along with the other mixtures.

This system differs from methods in vogue in which bactericidal action is determined by comparative counts of colonies developing from mixtures of a bacterial suspension and varying quantities of serum. When a serum also agglutinates the organism tested, methods dependent on colony counts must yield often fallacious results, and the complete sterilisation of a suspension containing a known number of bacteria by a known quantity of serum

*Bactericidal Action of Serum*Table I. *B. typhosus and human serum.*

Bacterial dilutions	1	2	3	4	5	6
	<i>S</i> /1	<i>S</i> /10 <sup>2</sup>	<i>S</i> /10 <sup>4</sup>	<i>S</i> /10 <sup>6</sup>	<i>S</i> /10 <sup>8</sup>	<i>S</i> /10 <sup>10</sup>
Control	+++	+++	++	+	f.c.	-
Bacteria + serum	+++	-	-	-	-	-

Bactericidal effect stated as +8.

Table II. *B. pyocyaneus and human serum.*

Bacterial dilutions	1	2	3	4	5	6
Control	+++	+++	++	f.c.	-	-
Bacteria + serum	+++	+++	++	f.c.	-	-

No bactericidal effect.

Table III. *B. typhosus and rabbit serum.*

	Bacterial dilutions	1	2	3	4	5	6
Before incubation	Control	+++	+++	++	+	f.c.	-
	Bacteria + serum	+++	+++	++	+	f.c.	-
After incubation (3 hours)	Control	+++	+++	++	+	f.c.	-
	Bacteria + serum	+++	++	+	-	-	-

Bactericidal effect stated as +4; no appreciable alteration in the number of viable organisms in saline.

Table IV. *Staphylococcus aureus and rabbit serum.*

	Bacterial dilutions	1	2	3	4	5	6
Before incubation	Control	+++	+++	++	+	f.c.	-
	Bacteria + serum	+++	+++	++	+	f.c.	-
After incubation (3 hours)	Control	+++	+++	++	+	f.c.	-
	Bacteria + serum	+++	+++	++	f.c.	-	-

Bactericidal effect stated as +2; no appreciable alteration in number of viable organisms in saline.

Table V. *B. diphtheriae and guinea-pig serum.*

	Bacterial dilutions	1	2	3	4	5	6
Before incubation	Control	+++	++	-	-	-	-
	Bacteria + serum	+++	+++	++	+	f.c.	-
After incubation (3 hours)	Control	+++	-	-	-	-	-
	Bacteria + serum	+++	+++	++	++	+	f.c.

Increased viability of organisms on transfer from serum as compared with saline; after incubation, end-point higher in test than control series; diminution in number of viable organisms in control series; increase in number in test series; no bactericidal effect.

Table VI. *Streptococcus pyogenes and pig serum.*

	Bacterial dilution	1	2	3	4	5	6
Before incubation	Bacteria + serum	+++	++	+	-	-	-
After incubation (3 hours)	Bacteria + serum	+++	++	+	-	-	-

End-points in test series same before and after incubation; no bactericidal effect.

affords a more reliable index of bactericidal action. In the system we have adopted, instead of testing complete sterilisation by cultivating the whole volume of each mixture, the relative degree of sterilisation was ascertained by loop-transfers. Our experience of this technique has justified the validity of the results obtained by it.

At first the series of bacterial concentrations used for the test consisted of successive decimal dilutions prepared from a standard concentration determined by comparison with Brown's opacity standards. In order to elicit definite end-points in both the control and test series when a serum was strongly bactericidal, an extensive range of such decimal

dilutions was found necessary— $S/1$  to  $S/10^{10}$  ( $S$  = standard concentration). This series did not yield a distinctly visible gradation in the relative amounts of growth obtained on loop-transfer from two successive dilutions unless near the end-point. Centimal dilutions from the standard,  $S/1$ ,  $S/10^2$ ,  $S/10^4$ ,  $S/10^6$ ,  $S/10^8$ ,  $S/10^{10}$ , gave, as a rule, a distinct gradation in the successive growths and proved adequate for comparative tests. This series was therefore generally used; in certain cases, to determine lesser differences, a decimal series was adopted.

The initial standard was varied (according to trial tests) for different organisms, the object being to arrange the series so that an end-point was obtained in the control at the fourth or fifth dilution. For organisms of the *coli*-typhoid group Brown's opacity standard No. 2 represented a suitable initial suspension.

The mixtures of bacteria and serum were made in small sterile stoppered test-tubes. The volume of bacterial suspensions was usually 0.5 c.c. and of serum 0.15 c.c. Even minute amounts (e.g. 0.025 c.c.) of an active bactericidal serum yielded distinct results by the method described.

The serum was either from defibrinated or coagulated blood, all precautions being taken to obtain a sterile product, and was used within a few hours of the withdrawal of the blood.

The mixtures were incubated usually for 3 hours. In tests with rabbit serum and *B. typhosus* and certain other organisms it was found that the full effect was developed in this time and further incubation did not elicit appreciably greater killing.

The transfers were made with a loop of 4 mm. diameter and each loopful was stroked out on a marked division of the plate.

The degree of bactericidal action was recorded as follows: for example, if, in the control series, growth resulted from all concentrations up to the fifth ( $S/10^8$ ), while in the test series growth occurred only from the first ( $S/1$ ), the result (a bactericidal effect) was stated as + 8; if in the control the end-point was at the fifth dilution ( $S/10^8$ ) and in the test at the third ( $S/10^4$ ), the result was recorded as + 4, i.e. the difference between the indices of the two dilutions.

In the detailed records the relative amount of growth from each transfer was recorded by + signs and other symbols as follows: + + + + (a confluent growth on stroke inoculation), + + +, + +, +, f.c. (a few colonies).

## METHOD II.

In testing certain organisms by the above method (e.g. *Streptococci*, *Pneumococcus*, diphtheroid bacilli, *Pasteurella* group, etc.) which rapidly lost viability in saline, it was noted that growth often occurred from higher dilutions in the test series than the control. In the case of *B. diphtheriae*, a comparison of the results of loop-transfers from both series before and after incubation, showed that the effect was due to the following factors: (1) loss of viability of the organisms in saline suspension; (2) maintenance of viability in the presence of serum and even some degree of multiplication; (3) greater viability on transfer to culture medium from serum as compared with transfer from saline solution; thus a proportion of the individual bacteria in saline were incapable of multiplying on culture medium while the presence of serum rendered them viable (see Table V). In this case a bactericidal effect could be excluded and the action of the serum was actually growth-promoting, but in testing organisms which rapidly lost viability in saline solution, and, in fact, all organisms which gave negative results by Method I, transfers were made from the mixtures of serum and bacteria both before and after incubation and the respective end-points of growth compared (see Table VI). This involved a double set of transfers for each specimen of serum tested.

For estimating bactericidal effects in Method II, the transfers before incubation provided the control, and the same system of notation was used as in Method I.



ANALYSES OF THE MECHANISM OF NATURAL BACTERICIDAL  
ACTION BY SERUM.

As a preliminary to the analytical study which was the main object in view, an extensive survey was made of the natural bactericidal properties of the serum of various animal species. Specimens of ox, sheep, horse, pig, rabbit, rat, guinea-pig, pigeon and human serum were tested with organisms representative of various bacterial groups.

It is not intended in this communication to detail or discuss the observations made in the course of such survey. Some of the data elicited were confirmatory of observations recorded by previous workers on the subject; many of the results have opened up questions which are still being studied and require further investigation.

The serum of all these animal species exhibited in a specially marked degree and most uniform manner bactericidal properties towards certain organisms, exemplified by the typhoid-paratyphoid and dysentery groups and the cholera and paracholera vibrios. The bacterial types selected, therefore, for the enquiry recorded in this paper were drawn mainly from these groups.

For the analyses of the bactericidal mechanism, ox, sheep, pig, rabbit, horse, guinea-pig and human sera were used, and the following organisms were selected for the experiments: *B. typhosus* (strain "CB"), *B. dysenteriae* Shiga, *V. cholerae* (strains "Bombay" and "3134") and *B. proteus* X 19.

In all cases inactivation of the serum occurred at 55° C. within half an hour, and the bactericidal reactions with these organisms were apparently dependent on a principle analogous in thermolability to serum-complement. To determine whether a sensitising antibody-like agent plays an essential part in bactericidal action along with this labile principle (assumed to be complement), a series of tests were carried out as follows:

Living organisms from agar cultures (18-24 hours) were treated for 2 hours at 0° C. with a large volume of serum (usually one slope-culture to 10 c.c. serum), the object being to sensitise them, if an antibody were present in the serum, without fixation of complement or bacteriolysis resulting (see Mackie and Finkelstein, 1930); thereafter the organisms were separated by centrifuging and washed in saline solution; a suspension of standard density was then prepared. In the text and tables such organisms are described as "sensitised." Serum was also treated with heat-killed (65° C.) cultures for 2 hours at 0° C. with the object of absorbing and removing the antibody, if present, without affecting the complement; the serum was separated by centrifuging. For absorption the minimum amount of growth found sufficient in preliminary tests to inactivate the serum was used (usually 12 agar slope cultures to 5 c.c. serum). Serum treated in this way is described as "absorbed." A bactericidal effect on the "sensitised" organisms by "absorbed" serum (if inactive *per se* towards unsensitised organisms) was taken as evidence of the existence of a sensitising antibody in the serum. The actual tests were carried out by Method I described above and the necessary controls were included.

In many cases, the existence of a sensitising antibody was demonstrated in this way without difficulty or dubiety. This held for all sera tested with *B. typhosus* (Table VII). The thermostability of this antibody was estimated

Table VII.

Serum	<i>B. typhosus</i> ("CB")			<i>B. proteus</i> X 19		<i>B. dysenteriae</i> Shiga	
	Sensitised by unheated and heated (55° C.) serum	Bactericidal effect by heated serum <i>plus</i> absorbed serum		Sensitised by unheated and heated (55° C.) serum	Bactericidal effect by heated serum <i>plus</i> absorbed serum	Sensitised by unheated and heated (55° C.) serum	
Pig							
Ox	Do.	Do.		Do.	Do.	Do.	
Horse	Do.	Do.		Not sensitised by unheated or heated serum	Bactericidal effect by heated serum <i>plus</i> absorbed serum	Do.	
Sheep	Do.	Do.		Do.	Do.	Do.	
Rabbit	Do.	Bactericidal effect by heated serum <i>plus</i> absorbed serum		Do.	Do.	Usually not sensitised by unheated or heated serum; sometimes partially	Usually no bactericidal effect by heated serum <i>plus</i> absorbed serum; sometimes a partial effect
Human	Do.	Do.		Not sensitised by unheated serum	Do.	Not sensitised by unheated or heated serum	No bactericidal effect by heated serum <i>plus</i> absorbed serum
Guinea-pig	Do.	Do.				Sensitised by unheated and heated serum	
						Sensitised by unheated and heated serum	
						Sensitised by unheated and heated serum but effects variable	
<hr/>							
Serum	<i>V. cholerae</i> ("Bombay")			<i>V. cholerae</i> ("3134")			
	Sensitised by unheated and heated (55° C.) serum	Bactericidal effect by heated serum <i>plus</i> absorbed serum					
Pig							
Ox	Sensitised by unheated serum, not by heated serum (variable)	Bactericidal effect by heated serum <i>plus</i> absorbed serum (variable)		Sensitised by unheated and heated serum	Bactericidal effect by heated serum <i>plus</i> absorbed serum		
Horse	Do.	Do.		Do.	Do.		
Sheep	Usually not sensitised by unheated or heated serum	Usually no bactericidal effect by heated serum <i>plus</i> absorbed serum		Do.	Do.		
Rabbit	Do.	Do.					
Human	Do.	Do.					
Guinea-pig	Do.	Do.					

by "sensitising" organisms with serum which had been heated at varying temperatures; it was found to be stable usually at 55° C., completely or partially stable at 60° C. and entirely inactivated at 70° C. (Table VIII).

In those cases in which sensitisation occurred, serum heated at 55° C. along with "absorbed" serum also yielded the full bactericidal effect of the native serum, though neither was active *per se* (Tables VII and IX). The lytic effect was clearly due to an antibody-like agent stable at 55° C., absorbed from the serum or inactivated by the organisms at 0° C., and capable of sensitising the organisms to the thermolabile complement which was not bound by the organisms at 0° C.

Table VIII. *B. typhosus* "CB" and pig serum.

			Bactericidal effect
Bacteria (untreated)	+ serum (untreated)		+ 6
" (untreated)	+ " (absorbed)		0
" (sensitised with unheated serum)	+ " (absorbed)		+ 6
" (sensitised with serum, 55° C.)	+ " (absorbed)		+ 6
" (sensitised with serum, 60° C.)	+ " (absorbed)		+ 6
" (sensitised with serum, 70° C.)	+ " (absorbed)		0

(To obviate coagulation, serum was diluted 1 in 4 with saline before heating.)

Table IX. *B. typhosus* "CB" and ox serum.

			Bactericidal effect
Bacteria (untreated)	+ serum (untreated)		+ 4
" (untreated)	+ " (absorbed)		0
" (untreated)	+ " (heated 55° C.)		0
" (untreated)	+ " (heated 55° C.) + serum (absorbed)		+ 4
" (untreated)	+ " (heated 60° C.)		0
" (untreated)	+ " (heated 60° C.) + serum (absorbed)		+ 2
" (untreated)	+ " (heated 65° C.)		0
" (untreated)	+ " (heated 65° C.) + serum (absorbed)		0

Further tests of the thermostability of the antibody were made in which serum heated at varying temperatures above 55° C. was used as antibody and "absorbed" serum as complement. It was noted that inactivation occurred between 60° C. and 65° C. (Table IX). The antibody corresponded, therefore, in thermolability to the natural haemolytic antibodies and natural agglutinins, though more labile than "immune" antibodies (see Mackie and Finkelstein, 1930).

With *B. typhosus* and various animal sera, no difficulty was experienced in demonstrating a bactericidal antibody, but the results with other organisms varied (see Table VII). *B. proteus* gave results similar to those of *B. typhosus* in the case of ox and pig serum, but with sheep, rabbit, horse and human serum an antibody could not be demonstrated by "sensitising" the organism as in the other experiments, though serum heated at 55° C. along with absorbed serum produced a bactericidal effect equal to that of the native serum, each being individually inactive (Table X).

In certain cases an antibody could not be demonstrated either by "sensitisation" or by testing heated serum along with "absorbed" serum, and such

Table X. *B. proteus* X 19 and sheep serum.

		Bactericidal effect
Bacteria (untreated)	+ serum (untreated)	+ 6
" (untreated)	+ " (absorbed)	0
" (untreated)	+ " (heated 55° C.)	0
" (untreated)	+ " (heated 55° C.) + serum (absorbed)	+ 6
" (sensitised with unheated serum) +	" (untreated)	+ 6
" (sensitised with unheated serum) +	" (absorbed)	0

Table XI. *V. cholerae* "Bombay" and sheep serum.

		Bactericidal effect
Bacteria (untreated)	+ serum (untreated)	+ 10
" (untreated)	+ " (absorbed)	0
" (untreated)	+ " (heated 55° C.)	0
" (untreated)	+ " (heated 55° C.) + serum (absorbed)	0
" (sensitised with unheated serum) +	" (absorbed)	0
" (sensitised with serum 55° C.) +	" (absorbed)	0

results might have been taken to indicate that the bactericidal effect was due directly to complement and that "absorption" inactivated it (Table XI). In other cases partial sensitisation effects were noted and also bactericidal effects by heated serum *plus* absorbed serum falling short of the full action of the native serum. In some cases also sensitisation was produced by unheated serum but failed with serum heated at 55° C., while the full effect of the original serum resulted from the combined action of heated *plus* absorbed serum—an apparently contradictory result (Table XII).

With certain organisms and serum from particular animal species the results varied with individual samples of serum.

The general results (Table VII) seemed to indicate, however, that these bactericidal effects were due to antibody-like agents (stable at 55° C.) acting along with serum-complement. It was apparent that, in many cases, the failure to demonstrate an antibody was due to its inability to sensitise the organisms under the conditions of the experiment. Various modifications in the technique of sensitisation were tried, *e.g.* varying the time of treatment at 0° C. from 2 to 18 hours, varying the temperature of treatment from 0° C. to room temperature, varying the amount of serum used. None of these modifications overcame the failure of certain sera to sensitise particular organisms, *e.g.* sheep serum and *V. cholerae* ("Bombay").

In tests with pig serum and *B. typhosus* it was noted that the antibody, like other natural antibodies (see Mackie and Finkelstein, 1928, 1930), was resident mainly in the carbonic acid-insoluble fraction of the serum (Table XIII). Sensitisation was attempted with this serum-fraction where difficulty occurred in sensitising with whole serum, but without further success.

An attempt was also made to demonstrate in certain cases an antibody by filtering the serum through a Berkefeld filter with a view to separating it from complement in this way. It was shown originally by Muir and Browning (1909) that an immune body passes such filter while complement is non-

*Bactericidal Action of Serum*Table XII. *V. cholerae* "Bombay" and ox serum.

		Bactericidal effect
Bacteria (untreated)	+ serum (untreated)	+ 10
" (untreated)	+ " (absorbed)	0
" (untreated)	+ " (heated 55° C.)	0
" (untreated)	+ " (heated 55° C.) + serum (absorbed)	+ 10
" (sensitised with unheated serum)	+ " (absorbed)	+ 10
" (sensitised with serum 55° C.)	+ " (absorbed)	0

Table XIII. *B. typhosus* "CB" and pig serum.

	Bactericidal effect
Bacteria + serum (untreated)	+ 6
" + " (heated 55° C.)	0
" + " (absorbed)	0
" + " (heated 55° C.) + serum (absorbed)	+ 6
" + CO <sub>2</sub> -insol. fraction (heated 55° C.)	0
" + " (heated 55° C.) + serum (absorbed)	+ 8
" + CO <sub>2</sub> -sol. fraction (heated 55° C.)	0
" + " (heated 55° C.) + serum (absorbed)	+ 2

filterable. This method has been used by Yoshinara (1921, 1922) for demonstrating the natural haemolytic antibody of ox serum for guinea-pig erythrocytes. Filtered sheep serum was tested on *V. cholerae* along with "absorbed" serum, but proved inactive.

It will be noted from Table VII that this interference with sensitisation varied according to the organism and the animal species from which the serum was obtained. Pig serum gave definite sensitisation effects with all the organisms tested. While a sensitising effect towards a particular organism (*e.g.* *V. cholerae* "Bombay") could not be elicited with the serum of one species (*e.g.* sheep), another serum proved effective (*e.g.* ox).

Experiments were carried out in which organisms "sensitised" by ox serum were tested with absorbed sheep serum. The results showed that the negative effects with sheep serum were not due to absorption of complement in addition to the antibody (*v. supra*). Thus absorbed sheep serum produced a bactericidal effect on organisms sensitised with ox serum though inactive towards the same organism "sensitised" with sheep serum (Table XIV). The failure to sensitise depended on some factor affecting the antibody which varied with the organism and the serum tested.

In a later section of this paper it is shown how cultures of various organisms (especially after heating at high temperatures) contain an extracellular agent which interferes in a non-specific manner with bactericidal antibodies. The non-specific neutralising action of killed organisms on the bactericidal power of human serum as described by Georgevitch (1926) is probably of the same nature. This inhibitory substance can be removed from cultures by repeated washing with saline solution, and in this way it has been possible to show that the failure to demonstrate a lytic antibody in the experiments described above was due to such agent. This was well illustrated in further experiments with sheep serum and *B. dysenteriae* Shiga (Table XV) and *B. proteus*. When

Table XIV. *V. cholerae* "Bombay" with ox and sheep serum.

		Bactericidal effect
Bacteria (untreated)	+ ox serum (untreated)	+ 6
" (untreated)	+ sheep serum (untreated)	+ 4
" (untreated)	+ ox serum (absorbed)	0
" (untreated)	+ sheep serum (absorbed)	0
" (sensitised with ox serum)	+ ox serum (absorbed)	+ 8
" (sensitised with ox serum)	+ sheep serum (absorbed)	+ 8
" (sensitised with sheep serum)	+ ox serum (absorbed)	0
" (sensitised with sheep serum)	+ sheep serum (absorbed)	0

Table XV. *B. dysenteriae* Shiga and sheep serum.

		Bactericidal effect
Bacteria (untreated)	+ serum (untreated)	+ 8
" (untreated)	+ " (absorbed)	0
" (sensitised in usual way)	+ " (untreated)	+ 8
" (sensitised in usual way)	+ " (absorbed)	0
" (washed with saline and then sensitised)	+ " (untreated)	+ 8
" (washed with saline and then sensitised)	+ " (absorbed)	+ 6

the culture used for sensitisation was washed with saline beforehand, sensitisation could be effected whereas the unwashed organisms were not sensitised.

In the case of *V. cholerae* ("Bombay") and sheep serum, a somewhat different result was obtained: washing of the culture used for sensitisation did not alter the previous result, but when the culture used for absorption was thoroughly washed, the "absorbed" serum produced a bactericidal effect on the "sensitised" organisms whereas serum absorbed with unwashed culture was inactive (Table XVI). Analogous results were obtained when serum heated at 55° C. was used as antibody and tested along with "absorbed" serum.

Table XVI. *V. cholerae* "Bombay" and sheep serum.

		Bactericidal effect
Bacteria (untreated)	+ serum (untreated)	+ 10
" (untreated)	+ " (absorbed in usual way)	0
" (untreated)	+ " (absorbed with growth previously washed in saline)	0
" (sensitised in usual way)	+ " (untreated)	+ 8
" (sensitised in usual way)	+ " (absorbed in usual way)	0
" (sensitised in usual way)	+ " (absorbed with washed growth)	+ 6
" (washed with saline and then sensitised)	+ " (untreated)	+ 10
" (washed with saline and then sensitised)	+ " (absorbed in usual way)	0
" (washed with saline and then sensitised)	+ " (absorbed with washed growth)	+ 8

In the case of *B. dysenteriae* Shiga and *B. proteus* the inhibitory agent was derived from the living culture used for sensitisation; in the experiment with *V. cholerae* it was derived from the organisms used for absorption, probably diffusing into the serum and later in the actual test interfering with the action of the antibody. It has been shown that it has no action on the complement of the absorbed serum. The negative results with heated serum plus absorbed serum (e.g. sheep serum and *V. cholerae*) could also be explained by the presence of the inhibitory agent in the absorbed serum.

It is of further interest that the inhibitory agent referred to may inactivate a particular bactericidin in the serum of one animal species (sheep) though not in another (pig). This is also illustrated in the results given in Table XIV. While unable in some cases to inactivate the bactericidin in unheated serum, it may affect the heated serum (*e.g.* ox serum and *V. cholerae* "Bombay"—Table XII).

It is difficult to explain these various differences. It will be shown later how the bactericidins for certain organisms (*e.g.* *B. proteus*) are more susceptible to this neutralising agent than others (*e.g.* *V. cholerae*). In the former case there may be a greater combining affinity between the antibody and this substance so that the latter is completely fixed in the "absorbed" serum and none is left uncombined to interfere with bactericidal action in the ultimate test. In the case of the *V. cholerae* bactericidin, there may be a lesser combining affinity so that no neutralisation occurs in the "sensitisation" process (the amount of the inhibitory substance being small in relation to the quantity of serum) whereas a large amount is left free in the absorbed serum to interfere with bactericidal action in the actual test. The different susceptibility of a particular bactericidin according to the animal species from which it is derived and according to the physical state of the serum would suggest that some other factor in serum, possibly thermolabile and varying according to species, is concerned in the neutralisation phenomenon.

It may be noted also that two strains of the same species are not identical in regard to their production of this substance: *e.g.* *V. cholerae* "Bombay" and "3134." The latter was readily sensitised with unheated sheep serum, and heated serum *plus* absorbed serum yielded the bactericidal action of the native serum (Table VII).

By removing from cultures used for sensitisation and absorption this extracellular inhibitory agent, it has been possible to demonstrate sensitising bactericidal antibodies where otherwise their presence could not be proved.

#### *Interchangeability of the complements of different animals.*

It has been shown above (Table XIV) how the absorbed serum of the sheep acts as complement along with the natural bactericidal antibody of the ox for *V. cholerae*. A number of experiments were carried out to ascertain whether the complements of different species were interchangeable in these bactericidal reactions. In such tests the "absorbed" serum from one species was found capable of acting as complement with the natural antibody derived from another, irrespective of the species and the type of organism (Table XVII). This applied even when the untreated serum, which was used as a complement, proved inactive by itself towards the particular organism (Table XVIII). Such results suggest that lack of bactericidal action is due to deficiency in antibody rather than in complement.

Table XVII. *V. cholerae* "Bombay" with ox and rabbit serum.

		Bactericidal effect
Bacteria (untreated)	+ ox serum	+ 10
" (untreated)	+ rabbit serum	+ 10
" (untreated)	+ ox serum (absorbed)	0
" (untreated)	+ rabbit serum (absorbed)	0
" (sensitised with ox serum)	+ ox serum	+ 10
" (sensitised with ox serum)	+ rabbit serum	+ 10
" (sensitised with ox serum)	+ ox serum (absorbed)	+ 8
" (sensitised with ox serum)	+ rabbit serum (absorbed)	+ 8

Table XVIII. *B. typhosus* "CB" with pig and guinea-pig serum.

	Bactericidal effect
Bacteria + pig serum	+ 4
" + guinea-pig serum	0
" + pig serum (heated 55° C.)	0
" + guinea-pig serum (absorbed)	0
" + pig serum (55° C.) + guinea-pig serum (absorbed)	+ 4

*Absorption of serum by charcoal.*

It has been shown (Dunlop, 1928; Mackie and Finkelstein, 1930) how treatment of serum by charcoal under certain conditions removes natural complement-fixing antibodies without affecting the haemolytic complement. A series of experiments was carried out, in which the bactericidal properties of serum after such treatment with charcoal were tested with unsensitised and sensitised bacteria. In this way it was found that absorption with charcoal, according to the method described by Dunlop, generally inactivated the serum (tested with unsensitised bacteria), but the absorbed serum was also inactive with sensitised organisms. "Bactericidal" complement, unlike "haemolytic" complement was, therefore, absorbed by charcoal. This effect was not studied further, as charcoal absorption did not provide a means of removing selectively the bactericidal antibody as in the case of the complement-fixing substance, but the result quoted would indicate that different complements or complement-moieties are concerned in haemolysis and bacteriolysis respectively (see Muir and Browning, 1908).

REACTING POWER OF THE SERUM IN RELATION TO THE AGE  
OF THE ANIMAL.

It has been shown how the serum of young animals (*e.g.* rabbits 3 or 4 weeks from the time of birth) fail to exhibit certain natural immunity reactions which are characteristic of adult animals. A litter of five young rabbits, 14 days old, was tested for the bactericidal power of their serum towards *B. typhosus* and *V. cholerae*. The serum of each proved practically equal in action to that of the mother (+ 6). The bactericidal property was, therefore, well developed at this stage of life, unlike natural agglutination, though similar, in this respect, to the natural property of fixing complement along with bacterial antigens (see Mackie and Finkelstein, 1928, 1930; and Gibson, 1930).



OBSERVATIONS ON THE SPECIFICITY OF NATURAL  
BACTERICIDAL ANTIBODIES.

With the object of determining whether these natural bacteriolytic antibodies are specific substances, a series of absorption tests was carried out. Normal serum was treated with heat-killed cultures for 2 hours at 0° C. (as in the experiments described in a previous section), separated by centrifuging and then tested for its bactericidal effect on the organism used for absorption and on representative strains of other species. Preliminary tests were made to ascertain the amount of culture required to annul completely the bactericidal effect towards the homologous organism.

In the first tests carried out, results seemed to indicate that absorption was non-specific: treatment of serum with a particular bacterium annulled its bactericidal power for some entirely unrelated species, although its effect on other unrelated organisms remained unaltered. After a series of such tests, it became apparent that these non-specific effects pertained more to the bactericidins for some organisms than others. For example, irrespective of the organism used for absorption and the serum absorbed, the bactericidin for *B. proteus* X 19 was invariably affected non-specifically whereas the bactericidal effect on *B. typhosus* "CB" and *B. dysenteriae* Y was only annulled by treatment of serum with the homologous organism. As regards the bactericidins for other organisms, results varied. It was also noted that such non-specific effects were often more marked quantitatively (when varying doses of the absorbing organism were used) than the absorption of the homologous bactericidin. When the organisms used for absorption had been heated to 120° C., treatment of the serum resulted in the complete abolition of its bactericidal action for all species tested. Organisms which had been heated and then washed several times with a considerable volume of salt-solution did not exert this effect. Thus, the substance responsible for such non-specific action was found to be present in the fluid of the heated bacterial suspension, and apparently heating at high temperatures liberated it in large amount from the organisms. It seemed likely that this agent corresponded to that which interfered with sensitisation (*v. supra*), and subsequent absorption tests were carried out with heat-killed cultures which had been washed three times with salt solution. The amount of growth required to produce complete absorption of the particular lysin was considerable. The method used was as follows:

A 24 hours' growth on a 6 in. plate of nutrient agar was emulsified in a small volume of saline solution, and heated at 65° C. for 1 hour; it was then washed three times with a large volume of saline; after the final washing the growth was emulsified in the serum to be treated; in general the growth from one plate was required to absorb completely the bactericidin in 2 to 3 c.c. of serum and, in some cases, even this did not effect complete absorption (see Tables XXII, XXV, XXVI).

Absorption tests carried out in this way revealed the highly specific nature of these bactericidal agents. Tables XIX-XXIX give a number of illustrative

examples of the results of such tests. It will be noted that, occasionally, the bactericidal effect for certain organisms, *e.g.* *B. proteus* X 19, was completely or partially affected in a non-specific manner. It has been shown how the bactericidins for this and certain other organisms are susceptible to a non-specific effect by unwashed cultures, and it is not surprising that in using a large amount of culture for absorption, washing failed at times to eliminate this influence. In Tables XIX–XXII are shown results of tests in which a

Table XIX. *Horse serum.*

Serum	Bactericidal effects.					
	<i>B. typhosus</i> "CB"	<i>B. paratyphosus</i> B	<i>B. aertrycke</i>	<i>B. dysenteriae</i> Shiga	<i>B. dysenteriae</i> Y	<i>V. cholerae</i> "Bombay"
Untreated	+4	+6	+6	+8	+8	+6
Absorbed by <i>B. dysenteriae</i> Shiga	+4	+6	+6	0	+8	+6

Table XX. *Ox serum.*

Serum	Bactericidal effects.					
	<i>B. typhosus</i> "CB"	<i>B. dysenteriae</i> Shiga	<i>B. faecalis</i> <i>alkaligenes</i>	<i>Pneumobacillus</i>	<i>B. proteus</i> X 19	<i>V. cholerae</i> "Bombay"
Untreated	+8	+4	+8	+4	+8	+10
Absorbed by <i>B. proteus</i> X 19	+8	+4	+8	+4	0	+10

Table XXI. *Ox serum.*

Serum	Bactericidal effects.				
	<i>B. typhosus</i> "CB"	<i>B. paratyphosus</i> A	<i>B. dysenteriae</i> Y	<i>V. cholerae</i> "Bombay"	<i>V. cholerae</i> "3134"
Untreated	+2	+6	+10	+10	+6
Absorbed by <i>V. cholerae</i> "3134"	+2	+4	+10	+10	0

Table XXII. *Sheep serum.*

Serum	Bactericidal effects.						
	<i>B. typhosus</i> "CB"	<i>B. paratyphosus</i> A	<i>B. paratyphosus</i> B	<i>B. aertrycke</i>	<i>B. dysenteriae</i> Shiga	<i>B. dysenteriae</i> Y	<i>B. proteus</i> X 19
Untreated	+8	+10	+4	+6	+8	+8	+4
Absorbed by <i>B. dysenteriae</i> Shiga	+10	+10	+6	+6	+4	+8	+4

serum was absorbed with one type of organism and then tested with a number of others. The organisms chosen for testing were those which were actively killed by the particular serum. In Table XIX it is noted how absorption of horse serum with *B. dysenteriae* Shiga removed the bactericidin for this organism, but had no influence on the bactericidal action of the serum towards *B. typhosus*, *B. paratyphosus* B, *B. aertrycke*, *B. dysenteriae* Y and *V. cholerae*. In the experiment shown in Table XX absorption of ox serum with *B. proteus* X 19 completely annulled the bactericidal effect towards this organism but did not reduce the value of the serum for *B. typhosus*, *B. dysenteriae* Shiga, etc. Table XXI shows analogous results after absorption of ox serum with *V. cholerae* (strain "3134"), and it is of special interest how absorption of the serum while annulling its effect on this strain, had no influence on the bactericidal action towards another strain ("Bombay") of the same species. These two strains of *V. cholerae* were similar in general characters and were both

*Bactericidal Action of Serum*Table XXIII. *Ox serum.*

Bactericidal effects.		
Serum	<i>B. dysenteriae</i> Shiga	<i>V. cholerae</i> "Bombay"
Untreated	+8	+10
Absorbed by <i>B. dysenteriae</i> Shiga	+0	+10
Absorbed by <i>V. cholerae</i> "Bombay"	+8	0

Table XXIV. *Pig serum.*

Bactericidal effects.		
Serum	<i>B. typhosus</i> "CB"	<i>B. dysenteriae</i> Shiga
Untreated	+8	+4
Absorbed by <i>B. typhosus</i> "CB"	0	+4
Absorbed by <i>B. dysenteriae</i> Shiga	+8	0

Table XXV. *Pig serum.*

Bactericidal effects.							
Serum	<i>B. typhosus</i> "CB"	<i>B. paratyphosus</i> A	<i>B. paratyphosus</i> B	<i>B. dysenteriae</i> Shiga	<i>B. dysenteriae</i> Y	<i>B. proteus</i> X 19	<i>V. cholerae</i> "Bombay"
Untreated	+6	+10	+4	+10	+10	+4	+12
Absorbed by <i>B. paratyphosus</i> B	+8	+8	0	+10	+10	+6	+10
Absorbed by <i>B. dysenteriae</i> Shiga	+8	+8	+4	+2	+10	+6	+10

Table XXVI. *Sheep serum.*

	Bactericidal effects.							
Serum	<i>B. typhosus</i> "CB"	<i>B. para-typhosus</i> A	<i>B. para-typhosus</i> B	<i>B. aertrycke</i>	<i>B. dysen-teriae</i> Shiga	<i>B. dysen-teriae</i> Y	<i>B. proteus</i> X 19	<i>V. cholerae</i> "Bombay"
Untreated	+8	+6	+4	+6	+10	+ 8	+2	+10
Absorbed by <i>B. typhosus</i> "CB"	+2	+6	+4	+6	+10	+10	0	+10
Absorbed by <i>V. cholerae</i> "Bombay"	+8	+6	+6	+6	+10	+10	+2	0

Table XXVII. *Sheep serum.*

Bactericidal effects.							
Serum	<i>B. typhosus</i> "CB"	<i>B. paratyphosus</i> A	<i>B. paratyphosus</i> B	<i>B. aertrycke</i>	<i>B. dysenteriae</i> Shiga	<i>B. dysenteriae</i> Y	<i>V. cholerae</i> "Bombay"
Untreated	+4	+8	+2	+8	+10	+10	+6
Absorbed by <i>B. paratyphosus</i> A	0	0	+2	+8	+10	+10	+8
Absorbed by <i>B. dysenteriae</i> Y	+2	+8	+2	+8	+10	0	+8

Table XXVIII. *Sheep serum.*

Bactericidal effects.							
Serum	<i>B. typhosus</i> "CB"	<i>B. paratyphosus</i> A	<i>B. paratyphosus</i> B	<i>B. aertrycke</i>	<i>B. proteus</i> X 19	<i>V. cholerae</i> "Bombay"	<i>V. cholerae</i> "3134"
Untreated	+6	+8	+6	+10	+4	+8	+12
Absorbed by <i>B. paratyphosus</i> A	0	0	+6	+8	+4	+8	+12
Absorbed by <i>V. cholerae</i> "Bombay"	+6	+8	+6	+8	+2	0	+10

Table XXIX. *Ox serum.*

Bactericidal effects.								
Serum	<i>B. typhosus</i> "CB"	<i>B. typhosus</i> "Cole"	<i>B. paratyphosus</i> A	<i>B. paratyphosus</i> B	<i>B. dysenteriae</i> Shiga	<i>B. dysenteriae</i> Y	<i>B. aertrycke</i>	<i>V. cholerae</i> "3134"
Untreated	+4	+6	+4	+4	+6	+10	+8	+10
Absorbed by <i>B. typhosus</i> "CB"	0	+6	+4	+4	+6	+10	+8	+8
Absorbed by <i>B. typhosus</i> "Cole"	0	0	0	+4	+6	+10	+8	+8

agglutinated by an anti-cholera serum. In Table XXVIII it is shown how absorption of a serum with *V. cholerae* ("Bombay") had no effect on the bactericidal action towards *V. cholerae* ("3134")—the converse result to that of Table XXI. When these results were obtained, agglutinin-absorption tests were carried out with an immune serum for a known strain of *V. cholerae*. Such tests elicited a difference in antigenic structure between the strains, though they apparently contained constituents in common. The bactericidin-absorption tests recorded above demonstrated even more sharply the difference between these strains than the agglutinin-absorption reaction with an immune serum.

Table XXII illustrates results similar to those in the previous tables, but in this case absorption of the homologous bactericidin was only partial; no quantitative reduction of the bactericidal values from other organisms was noticeable.

Tables XXIII and XXIV illustrate the results of absorption of serum with two organisms, the absorbed serum being tested with each. The effects were completely specific.

Tables XXV–XXIX afford further illustrations of the results of absorption tests. Occasional non-specific effects were noted, possibly due to incomplete removal (by washing) of the inhibitory agent referred to above, but apart from such results absorption was highly specific. The bactericidal antibodies for even closely related organisms (with the exception of *B. typhosus* and *B. paratyphosus* A—Tables XXVII, XXVIII and XXIX), seemed to be sharply differentiated. Absorption of a serum with *B. typhosus* ("CB") did not abolish its bactericidal effect on another typhoid strain ("Cole") though absorption with the latter annulled its action on both strains and also *B. paratyphosus* A (Table XXIX). Agglutinin-absorption tests with an anti-typhoid serum elicited no difference in the reactions of these two typhoid strains.

Such results along with those obtained with the two strains of *V. cholerae* indicate further the high grade of specificity of such natural bactericidal antibodies—their specificity being even more restricted than that of immune agglutinins.

#### DISCUSSION.

This study of the mechanism involved in the bactericidal action of normal serum contributes further information regarding the occurrence and characteristics of natural antibodies.

The data elicited are of particular interest in correlation with those obtained in previous studies of natural complement-fixing and agglutinating antibodies for bacteria (see Mackie and Finkelstein, 1930; Gibson, 1930). These studies showed how the normal serum of various mammalian animals contain specific antibodies for a wide variety of bacterial antigens, different animal species varying as regards the occurrence and reactivity of these agents, and a remarkable multiplicity of such natural antibodies was demonstrated.

The present enquiry was instituted primarily to ascertain whether bactericidal action by normal serum is dependent on antibody-like principles, and whether these are specific. Bacteria vary profoundly in their apparent susceptibility to this effect, and our analytical observations have been limited, in the first place, to the examination of the most highly reactive types, *e.g.* typhoid-paratyphoid bacilli, *V. cholerae*, etc. As noted by earlier workers, the bactericidal action of serum for such organisms is annulled by heating at 55° C., and, as long recognised, serum-complement plays an essential part in the process. Our observations leave no doubt as to the inactivity of complement *per se*, and an antibody-like principle invariably acts as an intermediary agent. In short, the natural bactericidal reactions studied are definitely analogous in their mechanism to that of an immune serum. As proof of the antibody nature of these intermediary agents, we have taken their capacity to "sensitise" bacteria to the bactericidal action of serum-complement and their "absorption" by bacteria from serum of 0° C. Their degree of thermostability is interesting in comparison with that of other natural antibodies: they are stable at 55° C. and are only inactivated at 60°–65° C.; in this respect they correspond closely to natural haemolysins and agglutinins (see Mackie and Finkelstein, 1928, 1930; Gibson, 1930). They contrast, however, with immune antibodies which are stable at higher temperatures. They differ also from natural complement-fixing antibodies, which are labile at 55° C. or even lower temperatures (40°–50° C.). It is of particular interest that natural bactericidal and complement-fixing antibodies should differ; the bactericidal reaction involves combination of complement with the antigen *plus* antibody, and it might seem reasonable to assume that complement-fixation is due to the same antibody. The difference in thermostability would tend to contra-indicate this.

Our results in attempted sensitisation of certain bacteria (*e.g.* *V. cholerae*) with this type of antibody in the serum of particular animals (*e.g.* sheep) are specially noteworthy. The failure to sensitise might have led to the conclusion that, in such cases, complement acted without any intermediary agent. The general results obtained by testing a variety of organisms with sera from different animals indicated by analogy, if not by direct proof, that in all cases an antibody-like substance was part of the bactericidal mechanism. The demonstration of a neutralising or inhibitory substance in bacterial cultures affecting the antibody explained such failures to sensitise. This neutralisation varied according to the particular organism, and the animal species from which the serum was obtained. It constitutes a phenomenon of some interest and importance, and requires further investigation. The neutralising agent is liberated in large amount from cultures heated at high temperatures, it is markedly thermostable (at 120° C.) and is not apparently related to the specific antigen of the organism in view of its non-specific action. It was shown by Georgevitch (1926) that killed organisms incubated with serum neutralised non-specifically the bactericidal action of the serum, and it seems

possible that the inhibitory effect we have noted corresponds to that described by this author. It is noteworthy that the neutralising substance acts at 0° C., but we have found it is more active at 37° C. This agent, irrespective of the organism from which it is produced or the serum used for the bactericidal test, affects strongly the bactericidal antibodies for certain organisms, and is less active towards others. It can be removed by repeated washing of cultures with large volumes of saline solution, and this has rendered it possible to demonstrate sensitising antibodies when otherwise these would have been effectively obscured in analytical tests. The peculiar differences in the results of sensitisation experiments with washed and unwashed cultures of different organisms suggest that the neutralising substance has a varying combining affinity for different bactericidal antibodies. It may also inactivate heated serum, though it exerts no effect on the fresh serum; it may inactivate the serum of one species but not another. These facts suggest the possibility of some property in serum which is protective against this agent. Two strains of the same bacterial species may differ in their production of the substance. Such results all serve to illustrate the great complexity of the serum-reaction studied and the many factors involved.

The sensitising antibody of the bactericidal reaction was found to be resident mainly in the carbonic-acid-insoluble fraction of serum, and in this respect resembles other natural antibodies.

It was found to be present in the serum of young animals at a stage when other natural antibodies (*e.g.* haemolysins, agglutinins) are absent, though it corresponds in this respect to the natural antibody responsible for complement-fixation along with bacterial antigens (see Mackie and Finkelstein, 1928; 1930; Gibson, 1930).

The complements of different animal species were found to be interchangeable in these bactericidal reactions and certain observations recorded have led us to assume that differences in the bactericidal properties of sera towards particular organisms depend on variation in the antibody rather than the complement.

It is also of interest that, unlike haemolytic complement, the complement concerned in bactericidal action is absorbed by charcoal (see Dunlop, 1928; Mackie and Finkelstein, 1930). It was originally suggested by Muir and Browning (1908) that different moieties of complement are concerned in haemolysis and bacteriolysis respectively.

The absorption tests clearly prove the specificity of these natural antibodies and the results of such tests support the analogous observations in the case of natural complement-fixing and agglutinating antibodies (referred to above). It is noteworthy that in our initial absorption experiments the bactericidal antibody appeared to be a non-specific principle and the existence of a non-specific natural antibody-like principle has in fact been suggested by Browning (1927). This apparent non-specific absorption was found to be due to the neutralising agent previously encountered in sensitisation experi-

ments. When carefully washed growths were used for absorption, highly specific effects were obtained. It would appear that the specificity of these antibodies may be even more restricted than that of an immune antibody (agglutinin), as evidenced by tests with different strains of *B. typhosus* and *V. cholerae*. It has been suggested by Felix and Olitzki (1929) that the bactericidal action of an immune serum is closely related to the O-agglutinin. The differences noted between the specificity of natural bactericidal antibodies as compared with immune agglutinins may be dependent on the different types of antigenic constituents (and their corresponding antibodies) concerned in the two reactions respectively. This requires further investigation, but the results cited provide a specially marked illustration of the high degree of specificity of natural bactericidal antibodies.

Correlating the observations recorded in this paper with previous observations on natural antibacterial antibodies, we would suggest that the various types of immune antibacterial antibodies have their precursors specifically differentiated in the serum of normal animals, certain types appearing at a very early stage of life, others later, and that specific immunisation enhances the output of the particular antistubstance and at the same time leads to its increased stability. In general, immune antibodies cannot be regarded, therefore, as substances formed *de novo* in response to a special stimulus. It would be of great interest to ascertain how far the response to immunisation with a particular antigen depends on the pre-existing power of the tissues to form antibodies for this antigenic substance. The work we have recorded opens up, therefore, many questions of great theoretical and also practical importance in immunity, and the multiplicity of natural antibodies which can be inferred from our work is of the greatest interest from a biological as well as the immunological standpoint.

#### SUMMARY AND CONCLUSIONS.

1. An analytical study has been made of the mechanism of natural bactericidal action by the serum of various animals (ox, sheep, horse, rabbit, guinea-pig, rat, man) towards certain organisms (*B. typhosus*, *B. dysenteriae* Shiga, *B. proteus*, *V. cholerae*) exhibiting the maximum reactivity to this effect.

2. Serum-complement has no bactericidal action *per se*, and an antibody-like agent invariably acts as an intermediary agent, "sensitising" the particular organism to the action of the complement and capable of being "absorbed" by it from serum at 0° C.

3. This sensitising agent is stable at 55° C. but labile at 60°–65° C. In this respect it resembles natural haemolysins and agglutinins, but contrasts with the more stable immune antibodies and the more labile natural complement-fixing antibodies (for bacterial antigens). It is resident mainly in the carbonic-acid-insoluble fraction of the serum. It is present in the serum of young animals before certain other natural antibodies have developed.

4. Absorption tests demonstrate the high degree of specificity of these natural bactericidal antibodies for particular bacteria.

5. A non-specific extracellular substance occurs in bacterial cultures which may neutralise or inhibit these antibodies, and interfere with their sensitising action even at 0° C.

6. This substance is liberated in large amount in cultures heated at high temperatures (120° C). It can be removed by repeated washing of growths in saline solution. It may inactivate a bactericidal antibody in heated serum, though not in fresh unheated serum, and may inactivate a particular antibody in the serum of one animal species but not in another. Strains of bacteria vary in their production of this substance.

7. The observations submitted in this paper, correlated with previous studies of natural antibodies by the authors and others, indicate that immune antibodies have their precursors specifically differentiated in the serum of normal animals and that, in general, immune antibodies are not substances formed *de novo*.

## REFERENCES.

- BROWNING, C. H. (1927). *Brit. Med. J.* **2**, 978.  
 BUCHNER, H. (1889). *Centralbl. f. Bakteriol. Orig.* **5**, 817 and **6**, 1.  
 DUNLOP, E. M. (1928). *J. Pathol. and Bacteriol.* **31**, 769.  
 FELIX, A. and OLITZKI, T. (1929). *Brit. J. Exp. Pathol.* **10**, 26.  
 GEORGEVITCH, A. (1926). *Compt. Rend. Soc. de Biol.* **95**, 1099.  
 GIBSON, H. J. (1930). *J. Hygiene*, **30**, 337.  
 GORDON, J. and WORMALD, A. (1928). *J. Pathol. and Bacteriol.* **31**, 753.  
 GRUBER, M. and FUTAKI, K. (1907). *München. med. Wochenschr.* 249 and 2050.  
 KNORR, M. (1929). *Handbuch der Pathogenen Mikro-organismen*, **2**, 663. By Kolle, Kraus and Uhlenhuth. Jena.  
 LEDINGHAM, J. C. G. (1922). *Lancet*, **2**, 898.  
 MACKIE, T. J. and FINKELSTEIN, M. H. (1928). *J. Hygiene*, **28**, 172.  
 ——— (1930). *Ibid.* **30**, 1.  
 MACKIE, T. J. and WATSON, H. F. (1926). *J. Hygiene*, **25**, 176.  
 MUIR, R. and BROWNING, C. H. (1908). *J. Pathol. and Bacteriol.* **13**, 76.  
 ——— (1909). *Ibid.* **13**, 232.  
 NUTTALL, G. F. H. (1888). *Zeitschr. f. Hygiene*, **4**, 353.  
 PETTERSSON, A. (1926). *Zeitschr. f. Immunitäts.* **48**, 233.  
 ——— (1927-8). *Ibid.* **54**, 292.  
 SEIFFERT, G. (1912). *Deutsch. med. Wochenschr.* 305.  
 ——— (1917). *Ibid.* 362.  
 SELTER, H. (1918). *Zeitschr. f. Hygiene*, **86**, 313.  
 YOSHINARE, N. (1921). *J. Pathol. and Bacteriol.* **24**, 124.  
 ——— (1922). *Ibid.* **25**, 153.

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# THE RESULTS OF SOME QUANTITATIVE EXPERIMENTS ON THE SERUM PRECIPITATION REACTION.

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## INTRODUCTION.

THE work described in this paper is based, very largely, on the work of Dean and Webb (1926), and the methods used in the various experiments are similar to those described by them. The results are discussed in two parts. The first is concerned with the mixture of different specimens of anti-horse serum, and with the relationship of the antibody in one specimen to the antibody in another. The second part deals with the nature of the reaction between antigen and antibody in the serum precipitation reaction. The two parts are dependent on each other, and the subject of the second suggested itself whilst the work on the first was in progress. The order in which the work is described is mostly that in which it was done. Nevertheless, conclusions arrived at in one part are, in one or two cases, of importance in the consideration of the other part, and *vice versa*. A question as to the possibility of strengthening the ratio of an antiserum by the addition of another antiserum led to the earlier experiments.

The work was carried out in the Department of Pathology at Cambridge. To Prof. H. R. Dean I am indebted for advice and criticism.

All the antisera with the exception of the anti-goat serum used in the experiments to be described were made by the injection of normal horse serum into rabbits. All reactions took place at room temperature. 0.85 per cent. saline was the diluent in all cases.

## PART I. MIXED ANTISERA.

Dean and Webb (1926) showed that when horse serum is mixed with anti-horse serum, the most rapid particulation takes place when the ingredients are in definite proportions. The proportion of horse serum to antiserum yielding optimal results is expressed as the antigen-antibody ratio. An antiserum of which 20 c.c. react most rapidly with 1 c.c. of horse serum is said to have an antigen-antibody ratio of 1 to 20. Different specimens of antiserum have different ratios. This may be due to a qualitative or quantitative difference in the antibody content of the respective antisera. The following experiments show

that in the majority of cases a quantitative difference in the antibody content is responsible for these differences. Different antisera contain varying amounts of the same antibody. The ratio of an antiserum is entirely dependent on the antibody content, and is not influenced by the other constituents of the serum. The constituents of an antiserum, other than antibody, do seem to influence the speed of the reaction, but in no way affect the proportions of horse serum and antiserum necessary for optimal reactions (Part II). Two antisera of the same ratio may take widely varying times to react with horse serum, when the dilutions of the ingredients in the two experiments are alike. It might be thought that the differences in the ratios of antisera are due wholly, or in part, to the effects exerted by variations in the constituents of the respective sera. This would not appear to be so. The amount of antigen in 1 c.c. of horse serum always reacts with a definite amount of antibody, although varying volumes of different antisera may be necessary to provide that quantity of antibody. The term "antigen-antibody ratio" does not mean that 1 part of antigen reacts with so many parts of antibody; it means that 1 part of horse serum reacts with so many parts of antiserum. It is not known how many parts of antibody react with 1 part of antigen, using antigen and antibody in the strictest sense. However, antigen does react with antibody in definite proportions, although those proportions are not yet capable of determination.

*Exp. 1* (Table I). Horse serum in falling quantities titrated against anti-horse serum 1192 ZA used in a dilution of 1 in 10.

Table I.

	H.S. 1 in 500 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 12 min.
Tube				
H.S.C.				
10	1.0	1 in 500	—	—
9	0.95	1 in 526.3	1 to 52.6	—
8	0.9	1 in 555.6	1 to 55.6	2
7	0.85	1 in 588.2	1 to 58.8	1
6	0.8	1 in 625	1 to 62.5	3
5	0.7	1 in 714.3	1 to 71.4	—
4	0.6	1 in 833.3	1 to 83.33	—
3	0.5	1 in 1000	1 to 100	—
2	0.4	1 in 1250	1 to 125	—
1	0.3	1 in 1666.7	1 to 166.7	—
A.S.C.	—	—	A.S. only	—

Controls unaffected.

In such experiments we are in the habit of referring to the horse serum dilution in a tube as being the dilution of the 1 c.c. of horse serum dilution placed in the tube. Similarly with the antiserum dilution. Both these dilutions are doubled of course, when the 1 c.c. of each are mixed, but the relative proportions of the two remain unchanged.

This experiment is similar to *Exp. 2* described in Dean and Webb's paper (1926, p. 477). The tabulation is simpler, and not so full. The series in this case is a close one; the amounts of horse serum dilution falling by 0.05 c.c. in the upper tubes to allow a nearer approximation to the correct result. A previous rough test had been performed. Horse serum in falling quantities was titrated

against a constant amount of anti-horse serum (1192 ZA). The first column describes the tubes in the series; H.S.C. and A.S.C. are the horse serum and anti-horse serum controls respectively. The second column shows the volume of a 1 in 500 dilution of horse serum delivered into the respective tubes. The antiserum control received none. The volume in each tube was made up to 1 c.c., where necessary, by the addition of the appropriate amount of saline; 1 c.c. of saline was added to tube H.S.C. and 1 c.c. placed in A.S.C. The third column gives the dilution of horse serum which each tube now contained. The fourth column shows the proportion of horse serum to antiserum in each tube after the addition of 1 c.c. of a 1 in 10 dilution of antiserum to every tube except H.S.C. Each tube now had a volume of 2 c.c. The time of the addition of antiserum was noted. The fifth and last column registers the order of the degree of particulation, and the time after which it was possible to determine this. Tube No. 7 was first, 8 second, and 6 third. No particulation occurred in the controls, even after many hours. It is seen that this antiserum reacted most rapidly with horse serum when the proportion of horse serum to antiserum was 1 to something between 55.6 and 58.8. If this antiserum is given an antigen-antibody ratio of 1 to 57, there is very little error.

The anti-horse serum from one rabbit may have a ratio of 1 to 20, that from another rabbit a ratio of 1 to 40. That is to say, with the same quantity of horse serum twice as much antiserum from one rabbit is needed to yield optimal results as from another rabbit. It might be thought that the antibody portion of one antiserum was different from the antibody portion of the other, or that the antigen was not the same in the two cases; one rabbit picking out one antigen and the other rabbit another. If the antibody in one antiserum is the same as the antibody in another, the difference in the ratios can be explained by saying there is more of this antibody in one than in the other. Assuming the antibody is the same:

If we mix equal parts of two anti-horse sera X and Y, of ratios 1 to  $x$ , and 1 to  $y$ , what should be the ratio of the resulting antiserum?

1 part of X reacts with  $\frac{1}{x}$  parts of horse serum (H.S.).

1    „    Y    „     $\frac{1}{y}$     „    „    „

$\therefore$  2 parts of mixed antiserum (A.S.) react with  $\frac{1}{x} + \frac{1}{y}$  parts of H.S.

i.e. 1 part of                    „                    „                     $\frac{1}{2x} + \frac{1}{2y}$                     „

i.e. 1    „                    „                    „                     $\frac{x+y}{2xy}$                     „

$\therefore$   $\frac{2xy}{x+y}$  parts                    „                    „                    1                    „

So the H.S. to A.S. ratio of the mixed antiserum is 1 to  $\frac{2xy}{x+y}$ .

*Exp. 2* (Table II). Horse serum titrated against a mixture of equal parts of two anti-horse sera, 1192 ZA and 1056 ZH. 1 c.c. of each antiserum were mixed, and the volume immediately made up to 20 c.c. The mixed antiserum is, therefore, in a dilution of 1 in 10.

Table II.

Tube	H.S. 1 in 350 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 15 min.
H.S.C.	1.0	1 in 350	—	—
10	1.0	1 in 350	1 to 35	—
9	0.95	1 in 368.4	1 to 36.8	—
8	0.9	1 in 388.9	1 to 38.9	3
7	0.85	1 in 411.6	1 to 41.2	1
6	0.8	1 in 437.5	1 to 43.75	2
5	0.75	1 in 466.7	1 to 46.7	—
4	0.7	1 in 500	1 to 50	—
3	0.6	1 in 583.3	1 to 58.3	—
2	0.5	1 in 700	1 to 70	—
1	0.4	1 in 875	1 to 87.5	—
A.S.C.	—	—	A.S. only	—

Table II is an example of an experiment in which normal horse serum in falling quantities is titrated against a constant quantity of mixed antiserum, made by mixing together equal parts of two anti-horse sera, 1192 ZA and 1056 ZH. 1192 ZA is the antiserum used in Table I, Exp. 1. The ratio of this antiserum has been determined as 1 to 57 (Exp. 1). The ratio of the second antiserum 1056 ZH was determined in a like manner, and found to be 1 to 34. Exp. 2 was carried out in exactly the same way as Exp. 1; the quantities, of course, differ. Before the experiment the calculated value was found for the mixed antiserum from the ratios of the component antisera, and a series of horse serum dilutions arranged accordingly. The equal parts of the component antisera were mixed, and immediately the dilution of 1 in 10 was made; this dilution showed a slight degree of opalescence. 1 c.c. of the antiserum dilution was at once delivered into each of the tubes except H.S.C. The antiserum control showed only a faint trace of opalescence, and no attempt at particulation, even after 24 hours. Tubes 7 and 6 were the first and second respectively with ratios 1 to 41.2 and 1 to 43.75. The ratio is taken as 1 to 42. Substituting the values of the ratios for the component antisera, 1192 ZA and 1056 ZH (1 to 57 and 1 to 34), in the given formula:

$$\text{Ratio is 1 to } \frac{2xy}{x+y}, \text{ we have 1 to } \frac{2 \times 57 \times 34}{57 + 34},$$

*i.e.* 1 to 42.6 as the ratio of the mixed serum; which agrees with the experimentally determined ratio of 1 to 42.

Table III is self explanatory. Ten different mixtures of two antisera in each were made from a series of ten antisera. These ten different antisera came from nine different rabbits. 1103 J and 1103 K were different bleedings of the same rabbit; they had very different ratios, as it happened, 1 to 35 and 1 to 15. This liability of the ratio of a rabbit's serum to change from bleeding to bleeding

was pointed out by Dean and Webb. These nine rabbits had been in the laboratory for longer or shorter periods, had various numbers of courses of injections, and various numbers of bleedings. Various specimens of horse serum had been used in the production of these antisera. Each rabbit had had horse serum from more than one horse at one time or another. The periods from the bleeding of the rabbits to the time of experiment varied very much, the

Table III. *Particulars of ten experiments with mixtures of two antisera in equal parts.*

No. of test	Particulars of component antisera		Mixed A.S.	
	A.S.	H.S. to A.S. ratio	H.S. to A.S. ratio	
			Experimental	Calculation
1	1056 ZH	1 to 34	1 to 42	1 to 42.6
	1192 ZA	1 to 57		
	1058 Q	1 to 19		
2	1103 J	1 to 35	1 to 26	1 to 24.6
	1058 Q	1 to 19		
	1195 F	1 to 62		
3	1103 J	1 to 35	1 to 29	1 to 29.1
	1195 F	1 to 62		
	1196 F	1 to 20		
4	1195 F	1 to 62	1 to 47	1 to 44.3
	1196 F	1 to 20		
	1105 I	1 to 37		
5	1195 F	1 to 62	1 to 29	1 to 30.2
	1105 I	1 to 37		
	1195 F	1 to 62		
6	1103 K	1 to 15	1 to 44	1 to 46.3
	1195 F	1 to 62		
	1103 K	1 to 15		
7	1398 A	1 to 28	1 to 20	1 to 19.5
	1308 D	1 to 41		
	1192 ZA	1 to 57		
8	1196 F	1 to 20	1 to 46	1 to 47.7
	1192 ZA	1 to 57		
	1196 F	1 to 20		
9	1308 D	1 to 41	1 to 27	1 to 26.9
	1103 K	1 to 15		
	1103 J	1 to 35		
10	1103 J	1 to 35	1 to 21	1 to 21

longest period was  $4\frac{1}{2}$  months, the shortest 2 months, *i.e.* sera of different ages were used. In fact it may almost be said that the antisera used were the result of all manner of doses and specimens of normal horse serum. The series did not include antisera which were either unusually quick or slow in precipitating. For this purpose an antiserum is defined as quick if, when it is diluted 1 in 10 and mixed with an optimal proportion of horse serum, particulation is evident within 10 minutes, and as slow if particulation has not taken place within  $1\frac{1}{2}$  hours.

It might be suggested that, in the case of two anti-horse sera *X* and *Y*, of ratios 1 to *x*, and 1 to *y*, the antibody of *X* is not necessarily the same as the

antibody of  $Y$ ; and that the antigen leading to the formation of the antibody of  $X$  is not necessarily the same as the antigen leading to the formation of the antibody of  $Y$ . If there were two antigens and two antibodies the following would result.

Suppose:

$\begin{smallmatrix} A & G \\ X \end{smallmatrix}$  is the antigen  $\longrightarrow$  antibody in  $X$

$\begin{smallmatrix} A & G \\ Y \end{smallmatrix}$  is the antigen  $\longrightarrow$  antibody in  $Y$

$\begin{smallmatrix} A & B \\ X \end{smallmatrix}$  is the antibody of  $X$ , and  $\begin{smallmatrix} A & B \\ Y \end{smallmatrix}$  is the antibody of  $Y$ .

Then the  $\begin{smallmatrix} A & G \\ X \end{smallmatrix}$  in one part of horse serum would react with the  $\begin{smallmatrix} A & B \\ X \end{smallmatrix}$  in  $x$  parts of  $X$ , and the  $\begin{smallmatrix} A & G \\ Y \end{smallmatrix}$  in the same part of horse serum would react with the  $\begin{smallmatrix} A & B \\ Y \end{smallmatrix}$  in  $y$  parts of  $Y$ .

Therefore to react with the  $\begin{smallmatrix} A & G \\ X \end{smallmatrix}$  and  $\begin{smallmatrix} A & G \\ Y \end{smallmatrix}$  in one part of horse serum,  $x$  parts of  $X$ , and  $y$  parts of  $Y$  would be required, *i.e.*  $x + y$  parts of antiserum in all, for there is no  $\begin{smallmatrix} A & B \\ X \end{smallmatrix}$  in  $Y$ , and no  $\begin{smallmatrix} A & B \\ Y \end{smallmatrix}$  in  $X$  (if they differ).

So no amount of mixed antiserum less than  $x + y$  parts could satisfy the antigens in 1 part of horse serum.

$$x + y \text{ is greater than } \frac{2xy}{x + y},$$

$$\therefore \frac{x^2 + 2xy + y^2}{x + y} = x + y,$$

and if anything less than  $x^2 + 2xy + y^2$  is divided by  $x + y$ , something less than  $x + y$  results; and  $2xy$  is obviously less than  $x^2 + 2xy + y^2$ .

So actually the antigen in 1 part of horse serum is satisfied by a less amount of antiserum than could possibly contain the necessary antibody, if there were two antigens and two antibodies. Experimentally 1 part of horse serum reacts with  $\frac{2xy}{x + y}$  parts of mixed antiserum. There cannot be two separate antigens and antibodies concerned in the reaction.

#### *Experiments with mixture of antigens and mixture of antibodies.*

*Exp. 3 (Table IV).* 0.5 c.c. of anti-horse serum 1196 F (ratio 1 to 20), and 1 c.c. of anti-goat serum 1295 B (ratio 1 to 40) were mixed, and the volume made up to 30 c.c. by the addition of saline, yielding a 1 in 20 dilution of mixed antiserum, a 1 in 60 dilution of anti-horse serum, and a 1 in 30 dilution of anti-goat serum. Twice as much anti-goat serum is needed to react with 1 c.c. of goat serum as anti-horse serum to react with 1 c.c. of horse serum. 0.5 c.c. of

horse serum and 0.5 c.c. of goat serum were mixed and the dilutions shown in column 2 arranged.

Table IV.

Tube	Mixed antigenic serum dilution	Mixed antigen to mixed A.S. ratio	Order of degree of particulation after 20 min.
Antigen C	1 in 500	—	—
12	1 in 500	1 to 25	—
11	1 in 555.6	1 to 27.8	2
10	1 in 600	1 to 30	1
9	1 in 666.7	1 to 33.3	3
8	1 in 750	1 to 37.5	—
7	1 in 857	1 to 42.8	—
6	1 in 1000	1 to 50	—
5	1 in 1200	1 to 60	—
4	1 in 1500	1 to 75	—
3	1 in 2000	1 to 100	—
2	1 in 3000	1 to 150	—
1	1 in 6000	1 to 300	—
A.S.C.	—	A.S. only	—

Controls unaffected.

Exp. 3, the one represented in Table IV, was a titration of mixed antigens against mixed antibodies. The antigens were horse serum and goat serum. The antibodies, obtained in both cases from rabbits, were anti-horse and anti-goat sera. Equal parts of horse serum and goat serum were mixed. The dilutions shown in the second column were obtained by making a series of dilutions from a primary 1 in 500 dilution, falling 0.5 c.c. at each step, and a series from a primary 1 in 600 dilution, also falling 0.5 c.c. at each step. The tubes used in the experiment were taken from the two series and arranged appropriately. Tube 10 was the optimal one, and in this tube both horse serum and goat serum are in a dilution of 1 in 1200. Anti-horse serum is in a dilution of 1 in 60; the horse serum to anti-horse serum ratio is, therefore, 1 to 20. Anti-goat serum is in a dilution of 1 in 30; the goat serum to anti-goat serum ratio is, therefore, 1 to 40. That is to say, horse serum and anti-horse serum are present in optimal proportions; similarly goat serum and anti-goat serum are present in optimal proportions. Now 1 c.c. of mixed antigen serum contains enough antigen to react completely with the antibody content of 10 c.c. of anti-horse serum, and of 20 c.c. of anti-goat serum; since 1 c.c. of mixed antigen serum contains 0.5 c.c. of both horse serum and goat serum. If the antigen content of 1 c.c. of horse serum, and of 1 c.c. of goat serum were contained in 1 c.c. of mixed antigen serum, then this 1 c.c. of mixed antigen serum would react with 20 c.c. of anti-horse serum plus 40 c.c. of anti-goat serum. Such a mixed antigen serum would have a ratio of 1 to 20 + 40, *i.e.* 1 to 60. This confirms the hypothesis that two separate antigens in an antigen serum react optimally with a mixed antiserum containing two different antibodies, when the ratio between antigen serum and antibody serum is 1 to  $x + y$ . Table IV shows that the optimal tube has a mixed antigen serum to mixed antiserum ratio of 1 to 30. This is an apparent anomaly, but 2 c.c. of mixed antigen serum are needed to furnish antigen to unite with 20 c.c. of anti-horse serum plus 40 c.c. of anti-goat serum. In the discussion as to whether there were two antigens and two anti-

bodies concerned in the titration of a mixture of antisera *X* and *Y*, the point was whether there were two antigens in the same 1 part of horse serum. The mixed antigen serum in this experiment is an artificially produced serum containing two antigens, and two parts of mixed serum are necessary to contain the antigens of 1 part of each of the components. If the antigens from 1 c.c. of both antigenic sera were contained in 1 c.c. of mixed antigenic serum, the ratio would be 1 to 60. A little thought will clear up this apparent anomaly.

*Exp. 4* (Table V). Horse serum titrated against a mixture of 0.5 c.c. of anti-horse serum 1196 F (ratio 1 to 20), and 1 c.c. of anti-goat serum 1295 B (ratio 1 to 40). The volume was made up to 30 c.c., so the anti-horse serum is present in a dilution of 1 in 60, and the anti-goat serum is present 1 in 30.

Table V.

Tube	H.S. 1 in 600 (c.c.)	H.S. dilution	H.S. to anti-H.S. ratio	Order of degree of particulation after 40 min.
H.S.C.	1.0	1 in 600	—	—
10	1.0	1 in 600	1 to 10	—
9	0.9	1 in 666	1 to 11.1	—
8	0.8	1 in 750	1 to 12.5	—
7	0.7	1 in 857	1 to 14.3	—
6	0.6	1 in 1000	1 to 16.7	2
5	0.5	1 in 1200	1 to 20	1
4	0.4	1 in 1500	1 to 25	3
3	0.3	1 in 2000	1 to 33.3	—
2	0.2	1 in 3000	1 to 50	—
1	0.1	1 in 6000	1 to 100	—
A.S.C.	—	—	A.S.	—

Controls unaffected.

In this experiment Tube 5 was the first. It contained horse serum in a dilution of 1 in 1200, and in the mixed antiserum dilution anti-horse serum is present 1 in 60, so the horse serum to anti-horse serum ratio is 1 to 20, showing that horse serum and anti-horse serum reacted in optimal proportions in spite of the presence of anti-goat serum. A corresponding experiment was performed in which goat serum alone was titrated against the mixture of anti-horse and anti-goat sera. The result was similar, giving goat serum to anti-goat serum ratio of 1 to 40.

*Exp. 5* (Table VI). Horse serum titrated against a mixture of equal parts of three anti-horse sera 1192 ZA, 1056 ZH, and 1103 K. 0.5 c.c. of each antiserum were mixed, and the volume immediately made up to 30 c.c. The mixed antiserum is therefore in a dilution of 1 in 20.

*Exp. 5*, Table VI, is an example of an experiment carried out on the same lines as the experiment of Table II. In *Exp. 5*, however, the antiserum is made up of equal parts of three different antisera, 1192 ZA, 1056 ZH and 1103 K. The ratios for these three antisera were respectively 1 to 57, 1 to 34, and 1 to 15. Here, again, the equal parts of antisera were mixed, and the volume immediately made up to 30 c.c. As there were 1.5 c.c. of mixed serum, the mixed anti-



*Serum Precipitation Reaction*

Table VI.

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 25 min.
H.S.C.	1.0	1 in 400	—	—
10	1.0	1 in 400	1 to 20	—
9	0.95	1 in 421.0	1 to 21	—
8	0.9	1 in 444.4	1 to 22.2	—
7	0.85	1 in 470.6	1 to 23.5	—
6	0.8	1 in 500	1 to 25	2
5	0.75	1 in 533.3	1 to 26.7	1
4	0.7	1 in 571.4	1 to 28.6	3
3	0.6	1 in 666.7	1 to 33.3	—
2	0.5	1 in 800	1 to 40	—
1	0.4	1 in 1000	1 to 50	—
A.S.C.	—	—	A.S. only	—

serum was in a dilution of 1 in 20. 1 c.c. of this dilution was at once delivered into each of the tubes (except H.S.C.) which already contained appropriate amounts of horse serum dilution. There was a faint opalescence in the antiserum dilution, but the antiserum control showed only the faintest trace, and did not particulate even after many hours.

Again assuming that the antibody is the same in these three antisera, if we mix equal parts of antisera X, Y, Z of ratios 1 to  $x$ , 1 to  $y$ , and 1 to  $z$  respectively, what is the ratio of the resulting antiserum?

1 part of A.S. X reacts with  $\frac{1}{x}$  parts of H.S.

1 „ A.S. Y „ „  $\frac{1}{y}$  „ „

1 „ A.S. Z „ „  $\frac{1}{z}$  „ „

Then 3 parts of mixed A.S. react with  $\frac{1}{x} + \frac{1}{y} + \frac{1}{z}$  parts of H.S.;

i.e. 1 part „ „ „  $\frac{1}{3x} + \frac{1}{3y} + \frac{1}{3z}$  „ „

i.e. 1 „ „ „  $\frac{xy + xz + yz}{3xyz}$  „ „

∴  $\frac{3xyz}{xy + xz + yz}$  parts of mixed A.S. react with 1 part of H.S.

∴ H.S. to A.S. ratio of mixed A.S. is 1 to  $\frac{3xyz}{xy + xz + yz}$ .

In Exp. 5 the ratios of the three best tubes were, in order, 1 to 26.7, 1 to 25, and 1 to 28.6. The ratio of 1 to 26 was given to the mixed antiserum.

Substituting the ratio values of the component antisera (57, 34, and 15) in the formula, H.S. to A.S. is

$$1 \text{ to } \frac{3xyz}{xy + xz + yz},$$

we have 
$$1 \text{ to } \frac{3 \times 57 \times 34 \times 15}{57 \times 34 + 57 \times 15 + 34 \times 15},$$

i.e. 1 to 26.7, which agrees with the experimentally determined ratio.

Table VII is self-explanatory. Five mixtures, each containing three antisera, were titrated, as in Table IV. These mixtures were made from amongst

Table VII. *Particulars of five experiments with mixtures of three antisera in equal parts.*

No. of test	Particulars of component antisera		Mixed A.S. H.S. to A.S. ratio	
	A.S.	H.S. to A.S. ratio	Experimental	Calculation
1	1192 ZA	1 to 57	1 to 26	1 to 26·7
	1103 K	1 to 15		
	1056 ZH	1 to 34		
2	1058 Q	1 to 19	1 to 33	1 to 30·8
	1195 F	1 to 62		
	1103 J	1 to 35		
3	1105 I	1 to 37	1 to 31	1 to 32·2
	1195 F	1 to 62		
	1196 E	1 to 20		
4	1056 ZH	1 to 34	1 to 36	1 to 36·3
	1192 ZA	1 to 57		
	1398 A	1 to 28		
5	1103 J	1 to 35	1 to 25	1 to 25·1
	1103 K	1 to 15		
	1308 D	1 to 41		

the ten antisera included in Table III; in addition another antiserum, 1196 E appears in Test 3 in Table VII. The previous remarks on this group of antisera apply equally whether they be used in mixtures of two or three.

*Exp. 6* (Table VIII). Horse serum titrated against a mixture of equal parts of ten anti-horse sera. 0·5 c.c. of each antiserum were mixed, and the volume was immediately made up to 100 c.c., *i.e.* 5 c.c. of the mixed antiserum in 100 c.c. The mixed antiserum is, therefore, in a dilution of 1 in 20.

Table VIII.

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of participation			
				H.S. A	H.S. B	H.S. C	Mixed H.S.
H.S.C.	1·0	1 in 400	—	—	—	—	—
12	1·0	1 in 400	1 to 20	—	—	—	—
11	0·95	1 in 421	1 to 21	—	—	—	—
10	0·9	1 in 444·4	1 to 22·2	—	—	—	—
9	0·85	1 in 470	1 to 23·5	—	—	—	—
8	0·8	1 in 500	1 to 25	—	—	—	—
7	0·75	1 in 533·3	1 to 26·7	1	1	1	1
6	0·7	1 in 571	1 to 28·6	—	—	—	—
5	0·65	1 in 616	1 to 30·8	—	—	—	—
4	0·6	1 in 666·7	1 to 33·3	—	—	—	—
3	0·5	1 in 800	1 to 40	—	—	—	—
2	0·4	1 in 1000	1 to 50	—	—	—	—
1	0·3	1 in 1333·3	1 to 66·7	—	—	—	—
A.S.C.	—	—	A.S. only	—	—	—	—

Controls unaffected.

In Table VIII are shown the results of four experiments, in each of which the antiserum used was a mixed one, made up of equal parts of ten antisera.

These ten antisera are the ones from which the antiserum mixtures were made in the earlier experiments. An eleventh antiserum, 1196 E, was used in Test 3, Table VII, but at the time of these final experiments this antiserum was used up, and so could not be included. The first three experiments (Table VIII) were titrations of three different specimens of normal horse serum *A*, *B*, and *C* against the antiserum mixture. In the fourth experiment a mixture of the horse sera *A*, *B*, and *C*, in equal parts, was titrated against the antiserum mixture. 1 c.c. of each horse serum were mixed, and immediately the volume made up to 60 c.c.; *i.e.* 3 c.c. in 60 c.c., representing a dilution of 1 in 20 of the mixed horse serum. The dilution of 1 in 400 was made from this. The results of the titrations are shown in the last four columns of the table. The tube with a ratio of 1 to 26.7 was the first in each experiment. The series was so close that further classification of the tubes was doubtful. This tube was the first in every case, whilst those next to it, on either side, were always ahead of the rest. If the ratio is given as 1 to 27, there is little error.

In order to work out a calculated value for the ratio of the mixed antiserum made from ten antisera, the following method was adopted:

1195 F	(1 to 62)	1 to 30.8	1 to 27.9
1058 Q	(1 to 19)		
1103 J	(1 to 35)		
1192 ZA	(1 to 57)	1 to 26.7	
1103 K	(1 to 15)		
1056 ZH	(1 to 34)		
1196 F	(1 to 20)	1 to 26.6	
1105 I	(1 to 37)		
1398 A	(1 to 28)		
1308 D	(1 to 41)		

The sera were arranged in groups of three, leaving the tenth one over. Dealing with each group in turn, and substituting values in the formula  $1 \text{ to } \frac{3xyz}{xy + xz + yz}$ , the value of the ratio of each group was obtained. This yielded three values, which in turn were treated in a like manner. A value for the ratio of the first nine antisera was the result. The above illustration explains the method. The tenth antiserum had to be brought in: If *A* parts of antiserum *X* (ratio 1 to *x*) are added to *B* parts of antiserum *Y* (ratio 1 to *y*), what is the ratio of the resulting mixed antiserum?

1 part of *X* reacts with  $\frac{1}{x}$  parts of H.S.

<i>A</i>	„	<i>X</i>	„	$\frac{A}{x}$	„	„
1	„	<i>Y</i>	„	$\frac{1}{y}$	„	„
<i>B</i>	„	<i>Y</i>	„	$\frac{B}{y}$	„	„

Therefore  $A + B$  parts of mixed A.S. react with  $\frac{A}{x} + \frac{B}{y}$  parts of H.S., *i.e.* with  $\frac{Ay + Bx}{xy}$  parts of H.S.

Therefore  $\frac{(A + B) xy}{Ay + Bx}$  parts of mixed A.S. react with 1 part of H.S.

Ratio of the A.S. is 1 to  $\frac{(A + B) xy}{Ay + Bx}$ .

We have nine parts of mixed antiserum, with a ratio of 1 to 27.9, and one part of the tenth antiserum, ratio 1 to 41. Substituting these values in the above formula we get:

Ratio is 1 to  $\frac{(9 + 1)(27.9 \times 41)}{9 \times 41 + 1 \times 27.9}$ , *i.e.* 1 to 28.8.

The experimentally determined ratio of 1 to 27 agrees well with the value found by the above calculation. The difference between the two figures is very slight; the calculation was made from ten ratios, which were not absolute in any case, although very nearly so. These four experiments again demonstrate that the normal serum from any horse gives the same results. Together with the experiments in which goat and anti-goat serum were concerned they show that when horse serum and anti-horse serum are present in optimal proportions, the reaction is not affected by the very various other constituents of the participating sera. The constituent substances, protein and otherwise, of horse and goat serum must be different in many ways, and the constituents of the serum must vary from horse to horse, and from rabbit to rabbit. A mixture of many sera, therefore, must contain an assortment of constituents differing very materially in many points from the constituents of a relatively simpler mixture of a single specimen of horse serum and one anti-horse serum. It is worthy of note that from amongst the very numerous constituents of the sera of nine rabbits and three horses, antigen and antibody continued to seek out each other and react in definite proportions. And when goat and horse serum together with their corresponding antisera were mixed, or when only one of these antigenic sera was present with both antisera, the individual antigens reacted with the corresponding antibodies in proportions which were unaffected by the presence of a complexity of other substances. These definite proportions were unaffected by any of the changes which were made in the constitution of the sera in the surrounding medium.

An examination of the results of the experiments shown in the foregoing tables shows that mixtures of antisera react with horse serum according to the formulae. These results are consistent with the idea that the antisera contain the same antibody. The calculated and experimental figures agree very well. The greatest difference between the two being 2.7 (in Test 4, Table III). No ratio assigned to an antiserum is absolute, although obviously it is very nearly so. The antiserum dilutions in the experiments varied; sometimes the dilution was 1 in 10, at other times 1 in 20. Different specimens of horse serum

were used in the course of the experiments. One specimen did not seem to differ from another. Dean and Webb pointed out that all normal horse sera behaved alike in experiments of this kind.

As they have become available other specimens of antiserum have been examined and have given confirmatory results.

*Exp. 7* (Table IX). A mixture of a quickly acting and a normally acting antiserum titrated against horse serum. Horse serum, in falling quantities, titrated against a mixed antiserum consisting of equal parts of anti-horse sera 1396 C (ratio 1 to 14) and 1193 G (ratio 1 to 35). The mixed antiserum dilution is 1 in 20.

Table IX.

	H.S. 1 in 300 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 25 min.
Tube				
H.S.C.	1.0	1 in 300	—	—
11	1.0	1 in 300	1 to 15	—
10	0.9	1 in 333.3	1 to 16.7	—
9	0.8	1 in 375	1 to 18.75	—
8	0.7	1 in 428	1 to 21.4	—
7	0.65	1 in 461	1 to 23.0	—
6	0.6	1 in 500	1 to 25	2
5	0.55	1 in 545	1 to 27.3	1
4	0.5	1 in 600	1 to 30	2
3	0.4	1 in 750	1 to 37.5	—
2	0.3	1 in 1000	1 to 50	—
1	0.2	1 in 1500	1 to 75	—
A.S.C.	—	—	—	—

Controls unaffected.

In the description of Table III it was stated that no very quick, or very slow antisera had been used. Occasionally an unusually quick acting antiserum is obtained. Two such antisera were on hand at one time, and the opportunity was taken of testing their behaviour in mixtures. Anti-horse serum 1396 C had a ratio of 1 to 14. When this antiserum in a dilution of 1 in 20 was titrated against falling quantities of horse serum, particulation was very advanced in many tubes in less than 5 minutes; so advanced as to be past accurate reading. Used in a dilution of 1 in 40, particulation took place in the optimal tube in about 15 minutes. This quickly acting antiserum was mixed with anti-horse serum 1193 G (ratio 1 to 35) in equal parts, and titrated against horse serum, as shown in Table IX, *Exp. 7*. Antiserum 1193 G had been previously used in mixtures with other antisera which were like those used in the earlier experiments, neither unusually quick, nor slow; it had behaved according to the formulae. Antiserum 1193 G was quite a normal antiserum. Substituting the values of the ratios of these two antisera in the formula, ratio is

$$1 \text{ to } \frac{2xy}{x+y}; \text{ we have } 1 \text{ to } \frac{2 \times 14 \times 35}{14 + 35}; \text{ i.e. } 1 \text{ to } 20.$$

A tube with a horse serum to mixed antiserum ratio of 1 to 27.3 was the first in the experiment, so this mixed antiserum was not obeying the formula. 0.5 c.c. of each of the component antisera was present in the 20 c.c. of mixed antiserum dilution. They were present in an individual dilution of 1 in 40. The ratio of

antiserum 1396 C, the quick one, was 1 to 14. 1 c.c. of a 1 in 40 dilution of this antiserum would be in optimal proportions when mixed with 1 c.c. of a 1 in 560 dilution of horse serum. The tube which particulated first in Table IX, Exp. 7, had a horse serum dilution of 1 in 545. It would appear that antiserum 1396 C was itself satisfying the antigen in that tube. Similar results were obtained when antiserum 1396 C was mixed with another antiserum, which was a normally acting one. Anti-horse serum 1308 F, with a ratio of 1 to 24, was the second very quickly acting antiserum. When mixed with normally acting antisera, it behaved as did antiserum 1396 C, and seemed to be itself satisfying the antigen. When the two very quickly acting antisera, 1396 C and 1308 F, were mixed in equal parts, and titrated against horse serum, the ratio of the optimal tube agreed with the ratio obtained by using the formula. This would seem to show that there is a difference between the antibody in an unusually quick acting antiserum and the antibody in the antisera which behave normally. It would appear that the antibody of the quickly acting antisera has a greater affinity for antigen than has the antibody of the normally acting antisera.

*Exp. 8 (Tables X and X A).* A mixture of a slowly acting and a normal antiserum titrated against horse serum.

Horse serum in varying amounts titrated against a mixed antiserum, consisting of equal parts of anti-horse sera 1196 G, a normally acting antiserum (ratio 1 to 29), and 1419 C, a slowly acting antiserum (ratio 1 to 10). Mixed antiserum dilution 1 in 10.

Table X. *Primary zone.*

Tube	H.S. 1 in 400 (c.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 45 min.
H.S.C.	1.0	1 in 400	—	—
10	1.0	1 in 400	1 to 40	—
9	0.9	1 in 444.4	1 to 44.4	—
8	0.85	1 in 470	1 to 47	—
7	0.8	1 in 500	1 to 50	—
6	0.75	1 in 533.3	1 to 53.3	1) Very close
5	0.7	1 in 571	1 to 57.1	2)
4	0.65	1 in 615	1 to 61.5	—
3	0.6	1 in 666.7	1 to 66.7	—
A.S.C.	—	—	A.S. only	—

Controls unaffected.

Table X A. *Secondary zone.*

Tube	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation after 50 min.
H.S.C.	1 in 10	—	—
1	1 in 10	1 to 1	—
2	1 in 20	1 to 2	—
3	1 in 30	1 to 3	—
4	1 in 40	1 to 4	2
5	1 in 50	1 to 5	1
6	1 in 60	1 to 6	—
7	1 in 70	1 to 7	—
8	1 in 80	1 to 8	—
A.S.C.	—	A.S. only	—

Controls unaffected.

Some antisera, used in a dilution of 1 in 10, react very slowly with horse serum. They will take from about 3 hours upwards to show particulation when mixed with horse serum in optimal proportions. Such slowly acting antisera usually have ratios less than the ratios of the antisera, which react within a more normal time. The ratio of a slowly acting antiserum is usually 1 to something less than 12. Anti-horse serum 1419 C was one of these slowly acting antisera, and its ratio was 1 to 10. When anti-horse serum 1196 G (ratio 1 to 29), a normally acting one, was mixed, in equal parts, with antiserum 1419 C, the resulting titration against horse serum showed a "double zone," such as those described by N. E. Goldsworthy (1928). There was an optimal tube with a ratio in the region of 1 to 50, and a second optimal tube with a ratio of 1 to something less than 10. Further tests showed that the ratios of the optimal tubes in the two zones were respectively 1 to 55, and 1 to 5. Tables X and X A outline the final tests on the two zones. The optimal tube of the zone in Table X particulated shortly before the optimal tube of the zone in Table X A.

In the primary zone, the one in Table X, if antiserum 1196 G were alone satisfying the antigen present, a tube containing a horse serum dilution of 1 in 580 would contain antigen and antibody in optimal proportions; for antiserum 1196 G is present in the mixed antiserum dilution in an individual dilution of 1 in 20. Experimentally the optimal point lay between Tubes 6 and 5. Tube 5 contained horse serum in a dilution of 1 in 571. It would appear that antiserum 1196 G, the one which reacted within a normal time, was itself satisfying the antigen in the primary zone.

At the moment I have no satisfactory explanation of the ratio of the optimal tube in Table X A. In the tubes of this experiment there is present such a large amount of horse serum that the content of adsorbable matter in the tubes cannot be regarded as constant, although the quantity of antiserum is constant. No doubt horse serum contains adsorbable matter, and where such a large amount of horse serum is present, it is likely that the concentration of adsorbable matter is varying considerably from tube to tube (see Part II). In the rest of the experiments in this part, and in Part II, the dilution of horse serum used is so great as to make the amount of adsorbable material furnished by the horse serum negligible. In all other experiments where antiserum is constant it has been quite proper to regard the adsorbable matter as constant. It is probable, therefore, that the figure for the ratio of the optimal tube in the secondary zone, viz. 1 to 5, is inaccurate to a fairly considerable extent. No "double zones" were found in the individual titrations of the constituent antisera, so it appears probable that the appearance of the secondary zone in the titration of the mixed antisera is due to the individual action of the antibody of antiserum 1419 C, the slowly acting one.

*Exp. 9 (Table XI).* A mixture of a degenerated and a normal antiserum titrated against horse serum.

Horse serum titrated against a mixture of equal parts of anti-horse serum

1308 E (ratio 1 to 50; previous ratio 1 to 40), and anti-horse serum 1196 F (ratio 1 to 20). Antiserum dilution 1 in 20.

Table XI.

Tube	H.S. 1 in 400 (o.c.)	H.S. dilution	H.S. to A.S. ratio	Order of degree of particulation
H.S.C.	1.0	1 in 400	—	—
10	1.0	1 in 400	1 to 20	—
9	0.9	1 in 444.4	1 to 22.2	—
8	0.8	1 in 500	1 to 25	3
7	0.7	1 in 571.4	1 to 28.6	1 { Zone. After 1 hour
6	0.6	1 in 666.7	1 to 33.3	2
5	0.5	1 in 800	1 to 40	—
4	0.4	1 in 1000	1 to 50	—
3	0.3	1 in 1333.3	1 to 66.7	2
2	0.2	1 in 2000	1 to 100	1 { Zone. After 1½ hours
1	0.1	1 in 4000	1 to 200	2
A.S.C.	—	—	A.S. only	—

Controls unaffected.

The ratio of an antiserum may change after a time. This alteration is always in one direction; the ratio changing to a greater figure, the antiserum becoming weaker. Thus within three weeks the ratio of anti-horse serum 1308 E was found to have changed from 1 to 40 to 1 to 50. This "degenerated" antiserum was mixed in equal parts with anti-horse serum 1196 F, and titrated against horse serum. Antiserum 1196 F (ratio 1 to 20) had maintained its ratio; it was one of the ten included in Table III. Table XI gives the results of the titration of the mixture of these two antisera. According to the formula, ratio is 1 to  $\frac{2xy}{x+y}$ , this mixed antiserum should have a ratio of 1 to 28.6. At the end of 1 hour, Tube 7, with a ratio of 1 to 28.6, was the leading tube. After 1½ hours another zone was apparent. Tube 2 was the optimal one in this secondary zone. The horse serum dilution in this tube was 1 in 2000, and the individual dilution of antiserum 1308 E (ratio 1 to 50) was 1 in 40; so Tube 2 contained horse serum and antiserum 1308 E in optimal proportions. It is possible that in Tube 2 the antibody of 1308 E was alone satisfying the antigen present. That the optimal tube of the primary zone was in agreement with the formula shows that the antibodies in the two antisera were reacting with the same antigen. The explanation of the appearance of a second zone, in which the antibody of antiserum 1308 E might be acting alone, is not certain. It may be that the antibody of 1308 E had a greater affinity for the antigen than had the antibody of 1196 F. In the optimal tube of the primary zone there was not enough of the antibody from 1308 E to satisfy the antigen present, and the antibody of 1196 F made up the deficiency. This experiment was repeated and the results confirmed. An experiment with another degenerated antiserum gave similar results.

No such secondary zones were found when mixtures of normally acting antisera, included in Table III, were titrated against horse serum. There was



no evidence that the antibodies of these antisera had different affinities for antigen.

The experiments with very slowly and very quickly acting antisera, and with degenerated ones, have not been extensive enough to justify the formation of definite conclusions. They do seem to indicate that the antibodies in these types vary in some way from the antibody in the majority of antisera (the normally acting ones). It is possible that the antibodies in the very slow and quick ones may be very different from that in the normal antisera. In the degenerated specimens the antibody appears to differ from the normal antibody only as regards affinity for antigen, which points to a slight difference in the structure of the two antibodies. The appearance of "double zones," such as those described by N. E. Goldsworthy, suggests the thought that zones appearing in the titration of a single specimen of antiserum may be due to the presence in that antiserum of more than one antibody; either of closely similar antibodies or of very different ones.

#### CONCLUSIONS.

1. The antibody in all the normally acting antisera produced in rabbits by the injection of normal horse serum reacts optimally in definite proportions with some antigen in normal horse serum, and these proportions do not appear to be influenced by other serum constituents.
2. There has been no evidence that the antibodies in different normally acting antisera have varying affinities for antigen.
3. It would appear, therefore, that the antibody in the normally acting antisera is always the same.
4. The antigenic content of different specimens of normal horse serum is apparently constant, qualitatively and quantitatively.
5. Abnormally acting antisera seem to contain antibodies differing from those present in the other types of antisera. The degree of difference may possibly be great or small.
6. The presence of more than one type of antibody in the same specimen of antiserum may explain the phenomena of "Zones."
7. The titration of mixtures of antisera against horse serum is a useful method of comparing the antibodies in the antisera.

#### PART II. THE NATURE OF THE REACTION BETWEEN ANTIGEN AND ANTIBODY IN THE SERUM PRECIPITATION REACTION.

The nature of the reaction between antigen and antibody has been a matter of contention since Ehrlich and Bordet propounded their different theories to explain this phenomenon. Ehrlich conceived the reaction as one between two substances following the laws which govern the ordinary chemical behaviour of crystalloids. Bordet (1903) sought to explain the quantitative discrepancies,

which occurred in experiments, by putting forward the adsorption theory. He maintained that the reaction between antigen and antibody was in every way analogous to colloidal reactions, which are governed by the laws of adsorption. The phenomena observable in the serum precipitation reactions seem to indicate that in the explanation of this reaction, at least, both theories contribute their part.

The experiments described in this paper appear to show that the serum precipitation reaction, as performed *in vitro*, consists of two different stages. The first stage is a reaction between antigen and antibody resulting in the formation of an antigen-antibody complex. This complex is adsorbent, and the second stage is the adsorption by the complex of some of the serum elements other than antigen and antibody. The first stage follows the laws of chemical equivalents, the second follows the laws of adsorption.

It would appear that when we follow the course of flocculation in a precipitation experiment, such as those described by Dean and Webb (1926), we are witnessing the adsorption of non-specific serum constituents by the antigen-antibody complex. The increase in size of the particles follows from the adsorption of more and more of the adsorbable material in the mixture, and from the fusion of the particles. The observation of the speed of particulation, and so of adsorption by the complex, serves as an index of the reaction between antigen and antibody. The speed of particulation is dependent on the amount of the complex, and upon the amount and nature of the adsorbable matter present.

#### THE FIRST STAGE.

##### *The reaction between antigen and antibody.*

Dean and Webb (1926) found that, when horse serum and anti-horse serum were mixed in optimal proportions, neither antigen nor antibody could be found in the supernatant fluid in appreciable quantity. When antibody was in excess the equivalent proportion of antibody was present in the supernatant fluid. If antigen were in excess only a small amount, if any, remained in the supernatant fluid; an amount bearing no relation to the equivalent amount. These workers state that the formation of a precipitate in a mixture of horse serum and anti-horse serum is delayed or inhibited, if either ingredient is in excess. Their experiments were performed by titrating a constant amount of antiserum against falling quantities of horse serum. If the reverse procedure is adopted, and the amount of antiserum is varied, whilst the horse serum is kept constant, a most interesting and instructive result follows. The tubes in which antibody is in excess do not, in this case, particulate after the one in which the ingredients are in optimal proportions, but the one containing the largest amount of antiserum is the first to show particulation.

*Exp. 1 (Table I). "Reverse" Experiment. (Horse serum constant.)*

Anti-horse serum 1193 E (ratio 1 to 34), in falling quantities titrated against a constant amount of horse serum in a dilution of 1 in 340.

Table I.

Tube	A.S. dilution	H.S. to A.S. ratio	Order of particulation
10	1 in 5.0	1 to 68	1 (after 7 min.)
9	1 in 5.5	1 to 61.2	2
8	1 in 6.25	1 to 54.4	3
7	1 in 7.14	1 to 47.6	4
6	1 in 8.33	1 to 40.8	5
5	1 in 10.0	1 to 34.0	6
4	1 in 12.5	1 to 27.2	7
3	1 in 16.7	1 to 20.4	8
2	1 in 25.0	1 to 13.6	9
1	1 in 50.0	1 to 6.8	10

Controls with saline and horse serum; and with saline and antiserum were unaffected.

Exp. 1, Table I, gives the particulars of such a "reverse" experiment. It was performed in the same way as the usual antigen-antibody titration, described by Dean and Webb, save that, in this case, the amount of antigen was constant, whilst the antibody was in falling quantities. The ratio of the antiserum, as determined by the usual method of titration, was 1 to 34; so that the tube in which antigen and antibody were in optimal proportions was No. 5. Tube 10 showed particles in the shortest time; it was followed by the ninth and eighth tubes in order, and so on down the series. Tube 5 took its place in this orderly progression of particulation. Tubes in the antigen excess region took a much longer time to show the reaction, and the further from the optimal a tube was placed, the less did the reaction proceed to completion. The fact that, in the tubes containing excess of antigen, the reaction does not go to completion, was still more apparent in an experiment in which the amount of antiserum was doubled in each succeeding tube; after 3 days the tubes containing excess of antigen had not precipitated completely, whilst those containing excess of antibody all reached complete precipitation in 2 hours.

Exp. 1 shows that antibody in excess has not delayed or inhibited the reaction. As the amount of antigen is constant in every tube the tubes vary from each other only in antiserum content. It might be suggested, as the speed of particulation has increased with the increase in the antiserum content, that increasing amounts of antibody have reacted with the antigen. That this is not so is proved by the excess of antibody, in equivalent amount, being traceable in the supernatant fluid. Dean and Webb (1926) pointed out that such excess could as a rule be found. With a view to confirming this observation the remaining experiments, described in this paper, were undertaken, and ample confirmation was obtained. The particular antiserum 1193 E used in Exp. 1 was used in experiments designed to demonstrate the excess of antibody in a supernatant fluid. Therefore, in all the tubes of Exp. 1, from 5 to 10 inclusive, a constant amount of antigen has reacted with a constant amount of antibody, yielding a constant amount of antigen-antibody complex.

The reaction between antigen and antibody does not seem to be an adsorption for the following reasons:

(1) If antigen were the adsorbent and antibody the adsorbable material, then, with a constant concentration of adsorbable antibody, we should expect

the amount of antibody adsorbed to increase logarithmically with increase in the amount of the adsorbent (antigen) (Freundlich, *Colloid and Capillary Chemistry*, p. 176, lines 5 and 6, English translation). Such conditions obtain in an experiment where varying quantities of antigen are titrated against a constant amount of antiserum, and so a constant amount of antibody—the usual titration performed by Dean and Webb (cf. Exp. 1, Part I). That the amount of antibody reacting with antigen has not increased logarithmically with increase in antigen is proved by the fact that excess of antibody is demonstrable in the supernatant fluid in equivalent amount. In the “reverse” experiments a constant amount of antigen has reacted always with the same quantity of antibody, despite increasing concentration of antibody.

(2) If antibody were adsorbent, and antigen were adsorbable material, we should expect a constant amount of antibody to adsorb increasing amounts of antigen with increasing antigen concentration. This increase should be logarithmic. Further the resulting antigen-antibody complexes would not be alike; the proportion of antigen to antibody in the complexes would increase with the increase in antigen concentration. Excess of antibody being traceable in the supernatant fluid in equivalent amount proves that a constant amount of antibody has not reacted with varying amounts of antigen, and that the antigen-antibody complex always contains antigen and antibody in the same relative proportions.

That antigen in excess is not traceable in the supernatant fluid does not contradict the preceding reasonings. Excess of antigen seems to be adsorbable by the antigen-antibody complex, and to exercise a protective action on the micellae of the complex, decreasing the sensitiveness of the complex towards the adsorbable matter present in the mixtures, and so preventing the reaction going to completion. An analogy of the adsorption of excess of one of the constituents in a chemical reaction by the resulting compound is furnished by the reaction between barium nitrate and sulphuric acid. In this case acid in excess is adsorbable by the resulting compound, and is not traceable in the supernatant fluid in equivalent amount.

Heidelberger and Kendall (1929), working with a non-protein antigen from pneumococcus and homologous antisera, have suggested that, in this case, the reaction between antigen and antibody is following a modification of the laws of mass action. They state that more than one type of complex is formed: one part of antibody being capable of reacting with one part of antigen to yield one type of complex, whilst one part of antibody may react with two parts of antigen to form a second type of complex, and so on. The finding of excess of antibody, in equivalent amount, in the supernatant fluid shows that the above conception does not explain the reaction between horse serum and anti-horse serum.

## THE SECOND STAGE.

*The adsorption by the antigen-antibody complex.*

In Exp. 1, Table I, the speed of particulation must depend on some variation in the antiserum content of the tubes, other than their antibody content. If we regard the antigen-antibody complex as adsorbent, and some of the constituents of the horse and anti-horse sera, other than antigen and antibody, as adsorbable materials, we find that the results of Exp. 1 are in accordance with the laws governing the velocity of adsorption in solution, viz. the initial velocity of adsorption is proportional to the concentration of adsorbable matter in the solution (cf. Freundlich, *Colloid and Capillary Chemistry*, p. 170, English translation). In Exp. 1, Tubes 5 to 10 inclusive contain a constant amount of the adsorbent antigen-antibody complex, and increasing amounts of adsorbable material in the form of antiserum, as distinct from antibody. The greater the concentration of antiserum, the greater has been the speed of particulation. "Reverse" experiments with eight different antisera yielded similar results. That the speed of particulation is proportional to the concentration of the adsorbable matter present in a mixture, is strikingly shown by the rest of the experiments to be described in this paper.

There is, therefore, this great difference in the behaviour of antigen and antibody. Antigen in excess is not left behind in the supernatant fluid in equivalent amount; it delays the speed of particulation, and prevents the reaction going to completion. Antibody in excess is found in the supernatant fluid, in equivalent amount, and excess does not prevent completion of precipitation. Antigen is of such a nature that, in excess, it exercises a protective action on the micellae of the complex, and decreases the sensitiveness of the complex towards the adsorbable matter present. No such protective action takes place when antibody is in excess.

Antigen and antibody react in equivalent proportions to produce definite amounts of the antigen-antibody complex. If the amount of the complex is constant in an experiment the speed of particulation is proportional to the amount of adsorbable matter present (Exp. 1, Table I).

The delay in particulation in the tubes containing excess of antibody in an experiment where a constant amount of antiserum is titrated against falling quantities of horse serum (the usual titration performed by Dean and Webb (cf. Exp. 1, Part I)), is not due to excess of antibody, but to the deficiency of antigen. There is not enough antigen to react with all the antibody present, and as the amount of antigen diminishes, so does the amount of the resulting antigen-antibody complex decrease. The concentration of antiserum, and so of adsorbable material, as distinct from antibody, is the same in every tube. The tube containing the largest amount of antigen-antibody complex, and no excess of antigen, is the first to particulate. This is the "Optimal Tube," in which antigen and antibody are present in the proportions necessary to react with each other completely, without excess of either. Thus we have this further

fact: the speed of particulation is dependent on the amount of the adsorbent antigen-antibody complex present in the mixture. The greater the amount of complex, the greater the speed of particulation.

#### EXPERIMENTS TO SHOW THE PRESENCE OF EXCESS OF ANTIBODY IN SUPERNATANT FLUIDS.

It is often found that two different anti-horse sera, having the same ratio and behaving normally in mixtures, take varying times to particulate, when they are mixed with horse serum in optimal proportions, and in the same concentrations. If a mixture of the two antisera is titrated against horse serum it can be shown that they both contain the same amount of the same antibody (Part I). Differences in the constituents of the antisera, other than their antibody content, appear to be responsible for the variation in the speed in particulation. These differences also affect the weights of the resulting precipitates. In Table VIII on p. 488 of Dean and Webb's paper (1926) are shown the weights of the resulting precipitates when two antisera of the same ratio were mixed in the same proportions with the same amount of horse serum. Both antisera had ratios of 1 to 50, yet the weight of precipitate in one case was about  $1\frac{1}{2}$  times the weight in the other. The antiserum containing the more adsorbable matter particulates the more rapidly. The relation between the speed of particulation and the amount of adsorbable matter present is most strikingly shown in experiments performed to demonstrate the presence of antibody in a supernatant fluid. If a quantity of horse serum is mixed with, say, twice the equivalent amount of antiserum, and after the completion of the reaction the whole is centrifuged and the supernatant fluid is decanted from the precipitate, it is possible to show the presence of the excess of antibody in the supernatant fluid. On mixing such a supernatant fluid with a further amount of antigen (horse serum), particulation may not take place for many hours, perhaps not overnight. Hartley (1925), with extracted sera, showed that when most of the serum elements, other than antigen and antibody, were removed, precipitation no longer occurred.

When falling quantities of horse serum are titrated against a supernatant fluid of this sort, the particulation may be so slow and the size of the particles so small, that it may be impossible to detect in which tube of a series the optimal particulation has occurred. The first reaction in the mixture has so depleted it of its content of adsorbable matter that, when the supernatant fluid is allowed to react with a further amount of antigen (horse serum), there is relatively so little adsorbable material present as to make the reaction very slow and difficult to read. However, if adsorbable matter is included in the mixture, the reaction is hastened and the size of the particles increased, so that the optimal tube is more easily picked out. Adsorbable matter may be added in varying ways. Normal rabbit serum may be added, although the content of adsorbable material varies greatly in different normal rabbit sera. The supernatant fluid may be mixed with a further amount of the antiserum, and

titrated against horse serum. These procedures provide adsorbable elements which hasten particulation and facilitate the reading of the result.

H. R. Dean (1912, 1916) showed that the addition of guinea-pig serum would increase the amount of precipitate, and Hartley (1925) used normal rabbit serum to increase the amount of precipitate.

*Exp. 2* (Table II). Titration of a supernatant fluid, with the addition of adsorbable material in the form of more antiserum.

10 c.c. of a 1 in 370 dilution of horse serum, and 10 c.c. of a 3 in 10 dilution of antiserum 1105 I (ratio 1 to 37) were mixed, and centrifuged after 16 hours. The supernatant fluid was decanted from the precipitate. 5 c.c. of the supernatant fluid were mixed with 5 c.c. of a 1 in 10 dilution of antiserum 1105 I, and titrated against falling quantities of horse serum.

Table II.

Tube	H.S. 1 in 300 (c.c.)	H.S. dilution	Order of degree of particulation After 1 hour 30 min.
10	1.0	1 in 300	—
9	0.9	1 in 333.3	—
8	0.8	1 in 375	1 } Very close
7	0.7	1 in 428	1 }
6	0.6	1 in 500	—
5	0.5	1 in 600	—
4	0.4	1 in 750	—
3	0.3	1 in 1000	—
2	0.2	1 in 1500	—
1	0.1	1 in 3000	—

The supernatant fluid was opalescent. Controls of horse serum and saline, and of the antiserum mixture and saline were set up. The antiserum control was faintly opalescent, but did not change even after many hours.

*Exp. 2*, Table II, is an example of an experiment, where adsorbable matter, in the form of further antiserum, is added to the supernatant fluid. A 3 in 10 dilution of anti-horse serum 1105 I was mixed with an equal amount of a 1 in 370 dilution of horse serum. The ratio of this antiserum, previously determined, was 1 to 37, so the mixture contained three times the antibody necessary to react with the antigen present. In the 20 c.c. of the primary mixture the dilution of the antiserum became 3 in 20. If only one of the three parts of antibody reacted with the antigen, the other two parts should remain in the supernatant fluid, in amount corresponding to an antiserum dilution of 2 in 20, or 1 in 10. On mixing equal parts of the supernatant fluid and a 1 in 10 dilution of the antiserum, there should result the equivalent of a 1 in 10 dilution of antiserum. A large part of the adsorbable matter had been removed during the first stage of the experiment, but that the supernatant still contained antibody in amount equal to a 1 in 10 dilution of the antiserum, i.e. the equivalent amount, was shown by the titration. It was impossible to decide between Tubes 8 and 7. A tube containing a 1 in 370 dilution of horse serum would react optimally with a 1 in 10 dilution of antiserum, and the experimental result of the titration agrees well with such figures. This experiment demonstrates that the excess of antibody remains in the supernatant fluid in equivalent amounts. Had the

supernatant fluid contained no antibody, then the dilution of the antiserum used in the titration would have been 1 in 20, and Tube 4 would have been expected to be the optimal one; if less than the equivalent amount had been present, a tube between 4 and 8 would have been optimal. The slight difference between the experimental and the calculated result, in that it was impossible to decide between Tubes 8 and 7, comes well within the limits of experimental error. It is open to objection that the volume of the supernatant fluid is not quite 20 c.c., but the error arising therefrom is so small as not to be of any importance.

*Exp. 3* (Table III). Titration of supernatant fluid without addition of adsorbable matter.

The supernatant fluid used in *Exp. 2*, Table II, was, without addition, titrated against falling amounts of horse serum.

Table III.

Tube	H.S. 1 in 300 (c.c.)	H.S. dilution	Order of degree of particulation after 7 hours
10	1.0	1 in 300	—
9	0.9	1 in 333.3	1 ) Very close
8	0.8	1 in 375	1 )
7	0.7	1 in 428	—
6	0.6	1 in 500	—
5	0.5	1 in 600	—
4	0.4	1 in 750	—
3	0.3	1 in 1000	—
2	0.2	1 in 1500	—
1	0.1	1 in 3000	—

Controls were set up, and remained unaffected.

In this experiment the supernatant fluid used in *Exp. 2*, having an antibody content equal to that of a 1 in 10 dilution of antiserum, was used without any addition. It was not possible to read the result until 7 hours had passed from the time the experiment was set up. Tubes 9 and 8 were the best ones, again indicating that the supernatant fluid contained the excess of antibody in equivalent amount. This experiment, although not readable until after 7 hours, was very much quicker than most of those in which supernatant fluid, without addition, was used.

*Exp. 4* (Table IV). Titration of supernatant fluid with the addition of more antiserum.

10 c.c. of a 1 in 370 dilution of horse serum, and 10 c.c. of a  $1\frac{1}{2}$  in 10 dilution of antiserum 1105 I (ratio 1 to 37) were mixed. After  $3\frac{1}{2}$  hours the mixture was centrifuged. To 9 c.c. of the supernatant fluid was added 1 c.c. of antiserum 1105 I, and a titration against falling quantities of horse serum performed.

The antibody content of the supernatant fluid in this case should be equal to that of a 1 in 40 dilution of the antiserum. When 1 c.c. of antiserum is added to 9 c.c. of the supernatant fluid the resulting 10 c.c. contain the equivalent of the antibody from 1.225 c.c. of antiserum, corresponding to a dilution of 1 in



*Serum Precipitation Reaction*

Table IV.

Tube	H.S. 1 in 270 (c.c.)	H.S. dilution	Order of degree of particulation after 1 hour 20 min.
10	1.0	1 in 270	—
9	0.95	1 in 284.2	2
8	0.9	1 in 300	1
7	0.85	1 in 318	3
6	0.8	1 in 337.5	—
5	0.75	1 in 360	—
4	0.7	1 in 385	—
3	0.6	1 in 450	—
2	0.5	1 in 540	—

Horse serum and antiserum controls unaffected.

8.16. The ratio of the antiserum is 1 to 37. The horse serum dilution to react optimally with this amount of antibody should be 1 in  $37 \times 8.16$ , *i.e.* 1 in 302. In this experiment it was possible to place the first three tubes in order of particulation. The dilution of horse serum, 1 in 300, in the optimal tube, No. 8, confirms the fact that the equivalent amount of the antibody excess is in the supernatant fluid. Without the addition of some adsorbable material to the supernatant fluid in this experiment it would have been most difficult, perhaps impossible, to determine the amount of antibody present with any degree of certainty. The amount of adsorbable matter left in the supernatant being so small, many hours would have passed before the particulation occurred, and the particles would have been so small as to make it practically impossible to get an accurate reading.

*Exp. 5* (Table V). Titration of supernatant fluid, with the addition of adsorbable material, in the form of normal rabbit serum.

10 c.c. of a 1 in 340 dilution of horse serum, and 10 c.c. of a 1 in 5 dilution of anti-horse serum 1193 E (ratio 1 to 34) were mixed, and centrifuged after 15 hours. To 10 c.c. of the supernatant fluid were added 2 c.c. of normal rabbit serum; the resulting 12 c.c. were titrated against falling amounts of horse serum.

Table V.

Tube	H.S. 1 in 600 (c.c.)	H.S. dilution	Order of degree of particulation after 1 hr. 50 min.
10	1.0	1 in 600	—
9	0.95	1 in 631	—
8	0.9	1 in 666.7	—
7	0.85	1 in 706	—
6	0.8	1 in 750	3
5	0.75	1 in 800	1
4	0.7	1 in 857	2
3	0.65	1 in 923	—
2	0.6	1 in 1000	—
1	0.5	1 in 1200	—

Controls were set up: one contained saline, normal rabbit serum, and horse serum; another held saline and horse serum; in the third were saline, supernatant fluid, and normal rabbit serum. They were unaffected.

The supernatant fluid should contain antibody equal in amount to a 1 in 20 dilution of antiserum 1193 E. The 12 c.c. of the mixed supernatant fluid and rabbit serum should contain antibody in amount equal to a 1 in 24 dilution of the antiserum. The ratio of the antiserum is 1 to 34. Therefore, the dilution of

horse serum containing the equivalent amount of antigen should be 1 in  $34 \times 24$ , i.e. 1 in 816. Tube 5, 1 in 800, was the first to particulate; Tube 4, 1 in 857 was the second, which demonstrates that the supernatant fluid contains antibody in amount equal to a 1 in 20 dilution of the antiserum. Another experiment, in which 5 c.c. of this supernatant fluid were added to 5 c.c. of a 1 in 10 dilution of the antiserum, was performed and the result in this case showed the presence of an equivalent amount of antibody in the supernatant fluid.

In another experiment the supernatant fluid, obtained by mixing less than the equivalent amount of horse serum with antiserum, was subjected to the action of a further amount of antigen, again less than the equivalent amount for the antibody in the fluid. The presence of the proportionate amount of antibody was demonstrable in the ultimate supernatant fluid. It was thought that this fluid would contain relatively so little matter other than antibody, that it might be possible to pass antibody through a collodion membrane. Three attempts were made with such fluids, but the presence of antibody could not be shown in the dialysates.

In 18 experiments, carried out in one or other of the ways described in Exps. 2 to 5, it was possible to demonstrate the presence of an equivalent amount of antibody in the supernatant fluid. Six different anti-horse sera were tested in the course of these experiments. The amount of the antiserum excess varied greatly. It was least in an experiment where one and an eighth times the equivalent quantity was mixed with horse serum, and greatest where three times the appropriate amount of antiserum was included.

A seventh antiserum, 1396 B, behaved differently. When twice the equivalent amount of the antiserum was mixed with horse serum, it was not possible to show the presence of any excess of antibody in the supernatant fluid. Not only was antibody not traceable in equivalent amount, there was apparently none at all. This experiment was repeated with the same result. When three times the proportionate amount of antiserum was used, antibody was found, but not in equivalent amount. Two parts should have been left in the supernatant fluid, the third part uniting with antigen. Only one part was traceable. This was repeated and confirmed. It seemed that, in the case of this particular antiserum, excess of antibody was removable by the precipitate, up to about the point where there was present twice the equivalent quantity; beyond this point excess was left in the supernatant fluid. Antiserum 1396 B was one which acted with unusual rapidity, so much so that it had been set aside from the others, used very sparingly, and generally regarded as a special one. By being so rapid it gave evidence of containing a great amount of adsorbable material. There seemed no reason to believe that, in this one case, antigen and antibody were not reacting in equivalent proportions. It is probable that this antiserum contained adsorbable matter in such unusually large amounts, as to cause the adsorption of excess of antibody up to a point.

The length of time elapsing, between the mixing of the ingredients and centrifuging, seemed to be of no importance in any of these experiments. The

shortest interval was  $1\frac{1}{2}$  hours, the longest 24 hours. No mixture was centrifuged until the reaction had apparently come to an end. The reaction was judged complete when flocculi seemed no longer to be settling on the precipitate. This does not mean that the mixtures were centrifuged immediately the reaction was complete. In many cases they were left for hours after the reaction was complete, quite often overnight.

The work described in this paper has been limited to the serum precipitation reaction. The results are not entirely in agreement with those of workers with other immunity reactions. The presence of an equivalent amount of antibody in the supernatant fluid is not in agreement with the "Danyasz Effect" (1902). The findings in the "Danyasz Effect" are approximated by those in the experiments with antiserum 1396 B, where excess of antibody was not traceable in equivalent amount. It is likely that the adsorbable matter in this antiserum may also be adsorbent. The antigen-antibody complex may adsorb the adsorbable matter, and the adsorbable matter may in turn adsorb excess of antibody. In the "Reverse" experiments, the results of titrating a constant amount of antigen against varying quantities of antibody do not coincide with the results of the titration of toxin and antitoxin by the Ramon (1923) method. Excess of antibody *per se* has not appeared to inhibit the serum precipitation reaction. Bordet's teaching, that the composition of the antigen-antibody compound varied according to the proportions of the two ingredients present, has not been confirmed. Dean and Webb (1926) found that excess of antigen was removed to a great extent by the precipitate. I do not think that this is proof that the excess of antigen has reacted with antibody. Excess of antigen may have been adsorbed by the antigen-antibody complex.

Whilst the present work was in progress a report by workers in America was noticed (Wu, Sah, and Li, 1929). They used iod-albumin as antigen, and by injecting it into rabbits obtained a homologous antiserum. Quantitative experiments revealed the amount of antigen present in the precipitates resulting from mixtures of antigen and antiserum in various proportions. Their results suggested that the reaction between antigen and antibody was chemical; agreeing with the conclusions arrived at in this paper.

In January 1930 in a private communication to Prof. H. R. Dean, Prof. W. W. C. Topley stated that Mr Tatham of his laboratory, following an examination of the weights of precipitates given in Dean and Webb's paper (1926, Table IX, p. 489) had formed the opinion that the figures were suggestive of some sort of adsorption process being concerned in the reaction. Mr Tatham considered that antigen and antibody combined according to chemical laws, and that subsequently the antigen-antibody compound adsorbed some of the excess of antibody which was present in the supernatant fluid. This conception of a specific adsorption of antibody is different from the non-specific adsorption of other serum elements which I believe to take place. The hypothesis of Mr Tatham has this in common with my own view that both assume a chemical union followed by a process of adsorption and both afford a basis of reconciliation between the theories of Ehrlich and Bordet. The

presence in the supernatant fluid of the equivalent amount of antibody disproves the theory that excess of antibody is adsorbed by the antigen-antibody complex.

#### CONCLUSIONS.

1. Antigen and antibody react in equivalent proportions, in the serum precipitation reaction, to form an antigen-antibody complex which is adsorbent.
2. The complex adsorbs suitable matter from the surrounding medium.
3. Excess of antigen is adsorbed by and exercises a protective action on the micellae of the complex, and prevents completion of the reaction. Antibody in excess does not affect the reaction in any way, but in the great majority of cases is traceable in the supernatant fluid, in equivalent amount.
4. The speed of particulation depends on the amount of complex and adsorbable matter present.
5. There is some evidence that in certain cases the nature and quantity of the adsorbable matter present may be such as to cause excess of antibody to be removed from the supernatant fluid. In such cases the adsorbable matter may possibly be adsorbent to the excess of antibody.
6. Antisera vary in the amount of antibody content, and in the amount and nature of the adsorbable matter they contain.
7. The addition of adsorbable matter is a useful way of hastening, and facilitating, the reading of reactions.

#### REFERENCES.

- BORDET, J. (1903). Sur le mode d'action des antitoxines sur les toxines. *Ann. Inst. Pasteur*, **17**, 161.
- DANYSZ, J. (1902). Contribution à l'étude des propriétés et de la nature des mélanges des toxines avec les antitoxines. *Ibid.* **16**, 331.
- DEAN, H. R. (1912). On the mechanism of complement fixation. *J. Hygiene*, **12**, 259.
- (1916). The influence of temperature on the fixation of complement. *J. Path. and Bact.* **21**, 193.
- DEAN, H. R. and WEBB, R. A. (1926). The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. *Ibid.* **29**, 473.
- EHRLICH, P. (1900). On immunity with special reference to cell life. *Proc. Roy. Soc.* **66**, 424.
- FREUNDLICH, H. (1926). *Colloid and Capillary Chemistry*. English translation. London.
- GOLDSWORTHY, N. E. (1928). The occurrence of multiple zones in the serum precipitation reaction. *J. Path. and Bact.* **31**, 525.
- HARTLEY, P. (1925). Observations on the rôle of the ether soluble constituents of serum in certain serological reactions. *Brit. J. Exp. Path.* **6**, 180.
- HEIDELBERGER, M. and KENDALL, F. E. (1929). A quantitative study of the precipitin reaction between Type III pneumococcus polysaccharide and purified homologous antibody. *J. Exp. Med.* **50**, 809.
- RAMON, G. (1923). La flocculation dans les mélanges de toxine et de serum antidiphthérique. *Ann. Inst. Pasteur*, **37**, 1001.
- WU, H., SAH, P. P. T. and LI, C. P. (1929). Composition of antigen-precipitate. *Proc. Soc. Exp. Biol. and Med.* **26**, 737.

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A NOTE ON THE CAUSE OF CERTAIN RED COLORATIONS ON SALTED HIDES AND A COMPARISON OF THE GROWTH AND SURVIVAL OF HALOPHILIC OR SALT-LOVING ORGANISMS AND SOME ORDINARY ORGANISMS OF DIRT AND PUTREFACTION ON MEDIA OF VARYING SALT CONCENTRATIONS.

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*(From the Laboratories of the British Leather Manufacturers' Research Association, Lister Institute.)*

(With 12 Text-figures.)

HIDES and calf skins cured with marine salts, especially if kept under conditions of warmth and moisture, tend to develop on the flesh side large areas of brick-red coloration. Similar red colorations have, for many years, been noted as occurring in similar conditions on salted fish, and a number of investigations have been made as to their cause, the first recorded being that of Farlow (1878). Following him were Mauriac (1886), Ewart (1887), Layet (1887), Le Dantec (1891) and, in recent years, Harrison and Kennedy (1922), Browne (1921-22) and Cloake (1923). All these workers have agreed in attributing the red coloration on salted fish to the growth of one or several species of red-coloured organisms, and the more recent workers (Harrison and Kennedy, Browne, Cloake) trace these organisms to the sea salt used in curing the fish, and find them to be chromogenic bacteria only capable of growth in a high concentration of salt. Clayton and Gibbs (1927) have examined many solar brines and salts, and have found that "they almost invariably contain micro-organisms which only exist and develop in the presence of abundance of common salt." Sturges and Heidemann (1923, 1924) have isolated many salt-loving organisms from meat-curing solutions, and Le Fèvre and Round (1919) from the brine of pickled cucumber.

Clayton and Gibbs found that stains on hides might be produced by salting with solar salts, and it was as part of some work undertaken with the object of finding whether certain extensive brick-red colorations on salted hides sent for examination were produced by true halophilic organisms coming from the curing salt or by the ordinary organisms of dust and dirt, that the experiments to be described were done.

A number of curing salts were examined for the presence of halophilic organisms, a solid fish-broth-rice-flour preparation of salt concentration of 25-30 per cent., modified from a fish-broth and rice formula suggested by

Clayton and Gibbs, being used as culture medium. The composition and preparation of the medium were as follows:

*Fish-broth.* 1 lb. of minced fresh cod was allowed to stand overnight in a litre of water. The resulting fluid was then strained through muslin and to it were added 30 per cent. of sodium chloride (British Drug Houses) and 0.01 per cent. of peptone. When the salt and peptone had dissolved the broth was again made up to 1 litre by the addition of tap water. The pH value was then adjusted to 8.

To make the solid medium for Petri plates, 30 gm. of rice flour were added slowly and with careful mixing to 100 c.c. of this broth. The resulting soft paste was poured onto Petri plates and autoclaved at 110° C. for 15 minutes. On this medium halophilic organisms grow freely, and such of the ordinary organisms of dirt and dust as have been examined do not grow. From all salts of marine origin examined, brick-red growths of apparently the same nature as those found on the hides were obtained, and red growths were produced on fresh hide pieces both by inoculation with red cultures obtained from the curing salts and by salting with the curing salts themselves.

The question whether halophilic organisms alone were to be held responsible for the red growths on the hides, or whether ordinary micro-organisms of dirt and dust, such as the coloured *sarcinae*, might also play a part, suggested an investigation into the capacity for growth and survival at different salt concentrations of the two types of organisms, and the following series of experiments was carried out.

Halophilic organisms giving brick-red growths were cultivated from four varieties of curing salt by sprinkling some of the salt on the surface of a Petri plate of the special medium, incubating in a moist atmosphere at 37° C. for about a week (these organisms grow slowly and grow best in the presence of a good deal of moisture), and sub-culturing, again on to the special medium, from the red colonies which developed. Films made from these red growths and stained with methylene blue showed, for the most part, large cocci, *sarcinal* in type and arrangement, but also a considerable amount of an amorphous material in which short, pleomorphic organisms, ill-defined in shape, could be vaguely made out. Later work has shown that there are at least two, probably many more, types of these organisms, one giving a dryish, wrinkled, red growth, and consisting of large cocci, which retain their shape when stained in the ordinary way with methylene blue, and another giving a moist colony, consisting of a rather clear, yellowish-pink jelly-like base, streaked and veined with the bright red colour, and breaking up into the amorphous material described above when rubbed up in a drop of water and stained with methylene blue. Some of the halophilic growths used in the experiments apparently contained more than one type of halophil, and no attempt was made to separate those growths into their component parts; they were used in the mixed condition.

Four series of slopes of the rice-flour-fish-broth medium, to which had been

added sodium chloride in concentrations of from 2–33 per cent., were prepared in small specimen tubes and strokes made on these from the halophilic growths obtained as described above from:

- (a) A salt from the Argentine.
- (b) A salt from Brazil.
- (c) A salt from New Zealand.
- (d) A salt from Bordeaux.

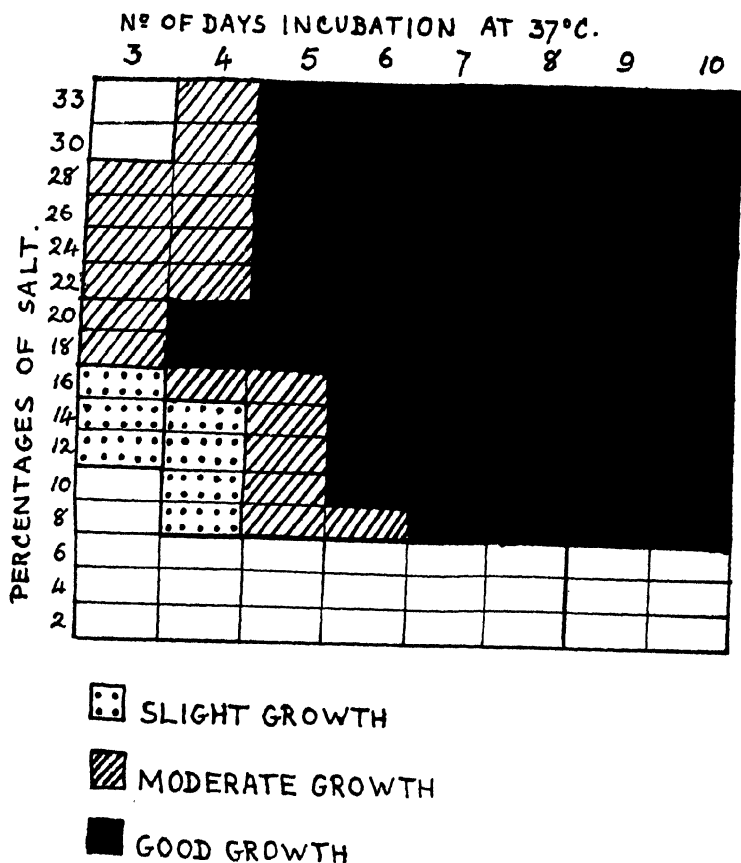


Fig. 1. Red culture from Argentine salt.

The inoculated tubes were kept over water in a covered vessel at a temperature of 37° C. and examined daily for growth. Ordinary laboratory agar slopes (0.5 per cent. sodium chloride) were also inoculated with these halophilic organisms and incubated at 37° C. but none showed any sign of growth. Figs. 1–4 indicate the manner in which growth proceeded and it will be seen that the heaviest growths were obtained at high salt concentrations and that no growth took place at salt concentrations below 6 per cent. (A blank space on the diagrams indicates “no growth.”)

The non-halophilic organisms selected for experiment were: (1) *Sarcina lutea*, (2) *S. aurantiaca*, (3) *S. rosea*, (4) *M. tetragenus*, (5) *Actinomyces*, (6) *B. proteus*, (7) *B. pyocyaneus*, (8) *B. fluorescens liquefaciens*. The first six were selected, as they had all been isolated by Stather and Liebscher (1929) from hide pieces showing the brick-red coloration described above. The last two were selected, as they are amongst the organisms giving rise to putrefaction, and the

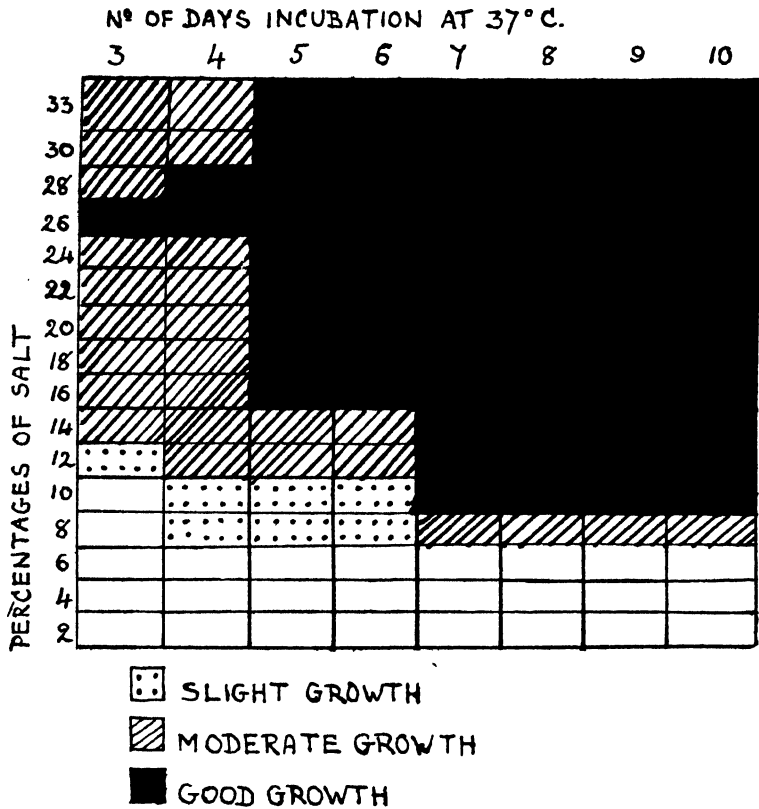


Fig. 2. Red culture from Sal Diamanté, Brazil salt.

question as to how far different degrees of salt concentration were likely to inhibit the growth of putrefactive organisms was also under consideration.

The procedure used with these organisms was rather different from that adopted with the halophils, as it was designed to find out not only whether any growth could take place at high salt concentrations, which could be ascertained by examining the broth tubes for turbidity, but also how long the organisms under investigation could remain alive under such conditions and be capable of development when removed to more favourable conditions. Sets of ordinary laboratory broth tubes were prepared with concentrations of sodium chloride ranging from 0.5-33 per cent. The pH value of these tubes in the range be-



tween 0.5-6 per cent. was 7, from 8-20 per cent. between 6.5 and 7, and from 20-33 per cent. approximately 6.5. The tubes were inoculated from 48-hour agar cultures of the organisms to be tested, and were kept at room temperature (approximately 20° C.). Sub-cultures were made daily from the salt broths onto ordinary agar slopes (0.5 per cent. NaCl), and the charts represent the readings obtained from these slopes. The experiment was not originally intended to measure the gradual decrease in numbers of organisms, but only to

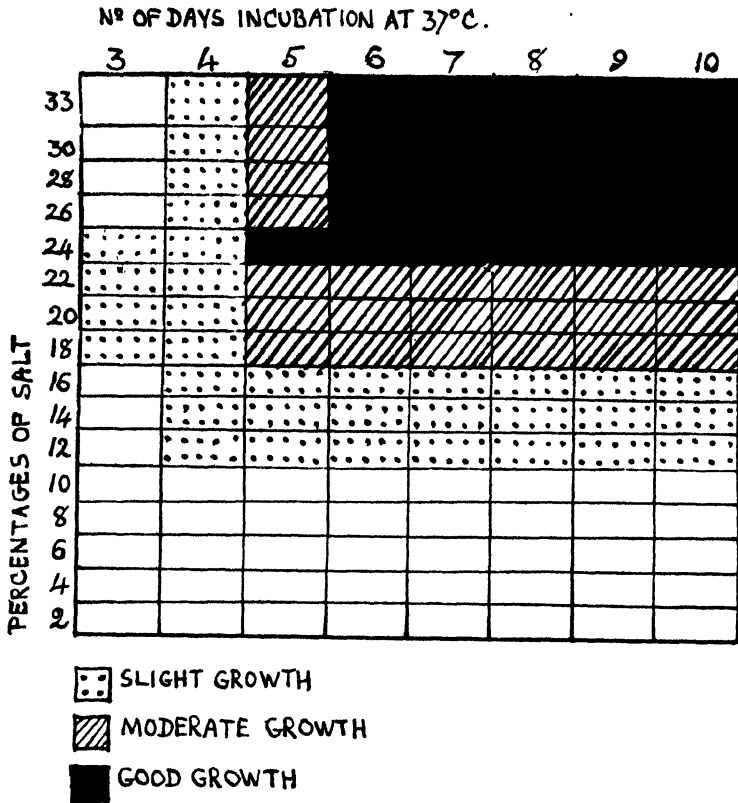


Fig. 3. Red culture from New Zealand salt.

ascertain how long they could survive at the different salt concentrations—hence the technique employed. It was found, however, that the amount of growth on the agar sub-cultures gave some information on this point and, throughout most of the experiment, care was taken by using the same platinum loop, and as nearly as could be judged the same amount of the salt broths in making the sub-cultures, to make the day to day comparisons of the amount of growth on the sub-cultures of some value as a measure of the rate of destruction of the organisms in the broth tubes. The sub-cultures were examined for growth at the end of 24 and 48 hours, and diagrams made from the results of

the 48 hours' observations. Figs. 5-12 show the results of these experiments. (A blank space on the diagrams indicates "no growth.")

It is clear from a comparison of the two sets of diagrams, making all allowances for accidental findings due to slight variations in technique or interpretation, that though there is overlapping of the two groups in the middle of the salt scale, as regards possibility of growth, their optimum growth conditions are widely different. At one end of the salt scale the halophils are incapable of

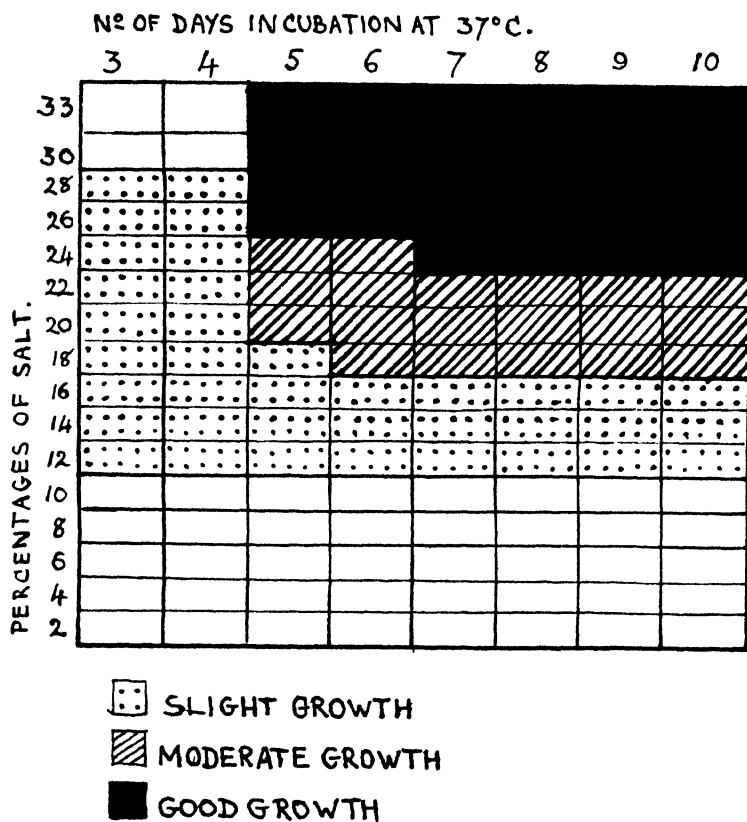
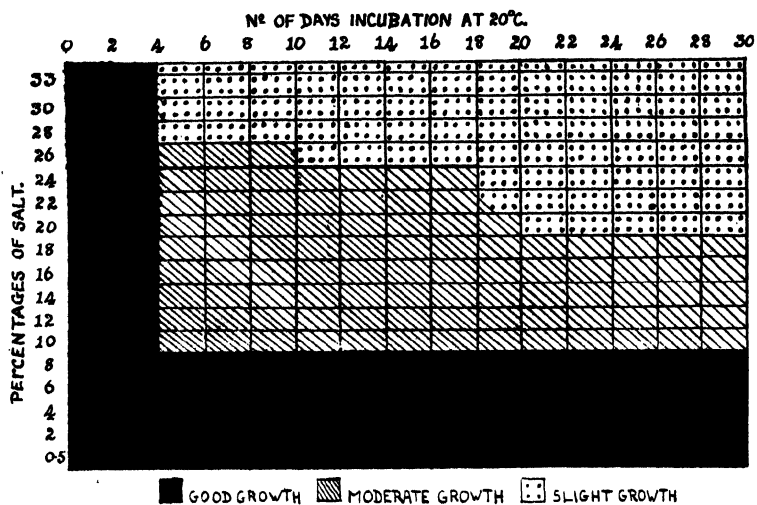
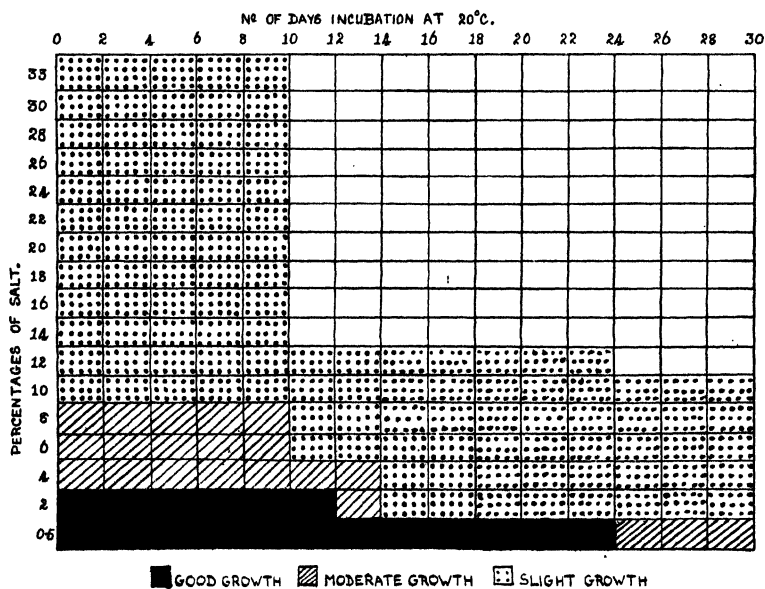


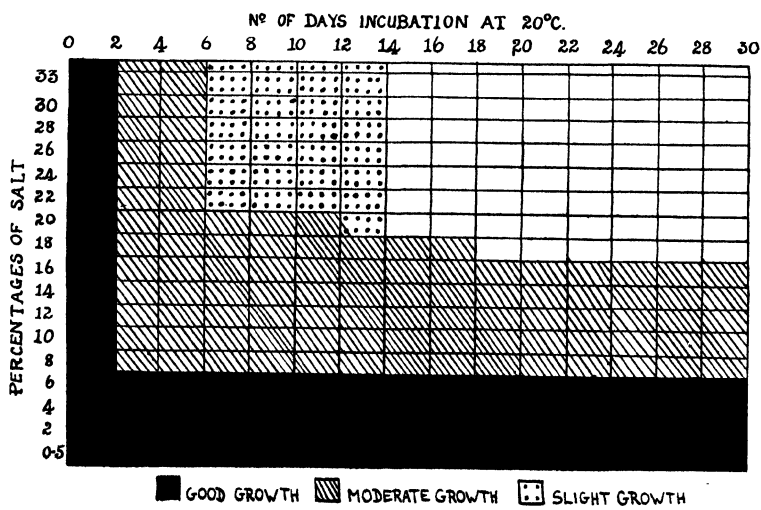
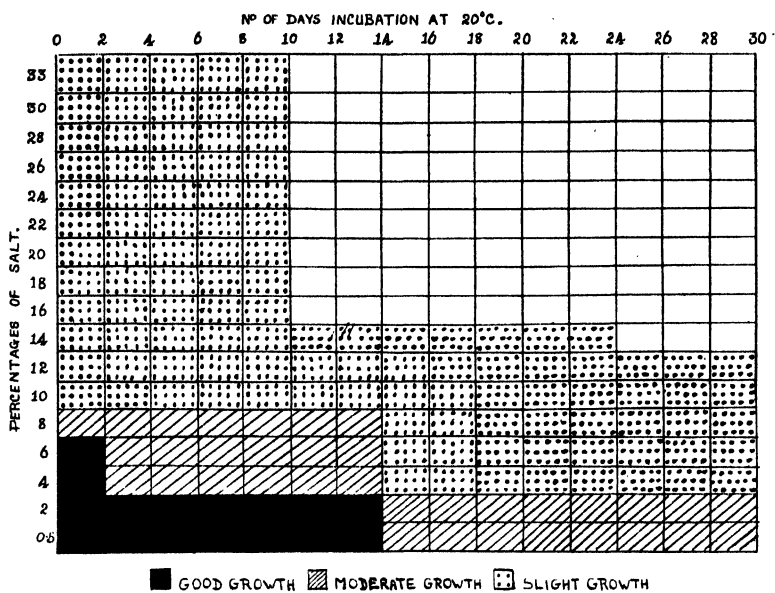
Fig. 4. Red culture from Bordeaux salt.

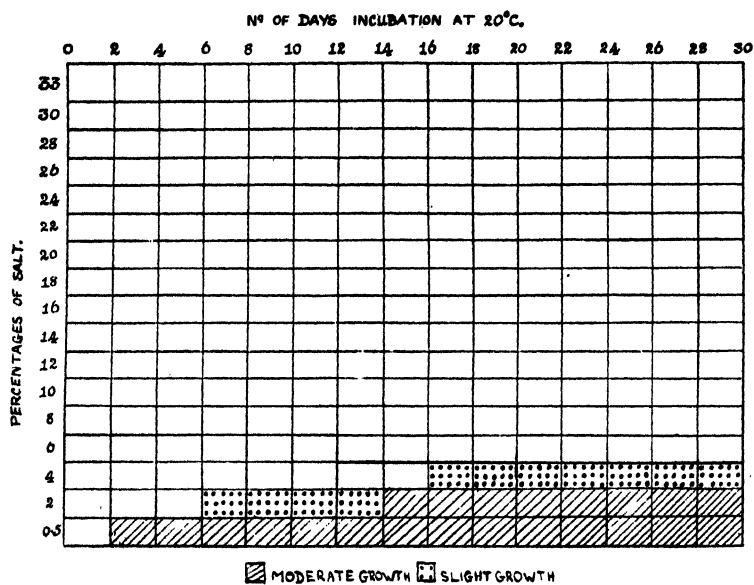
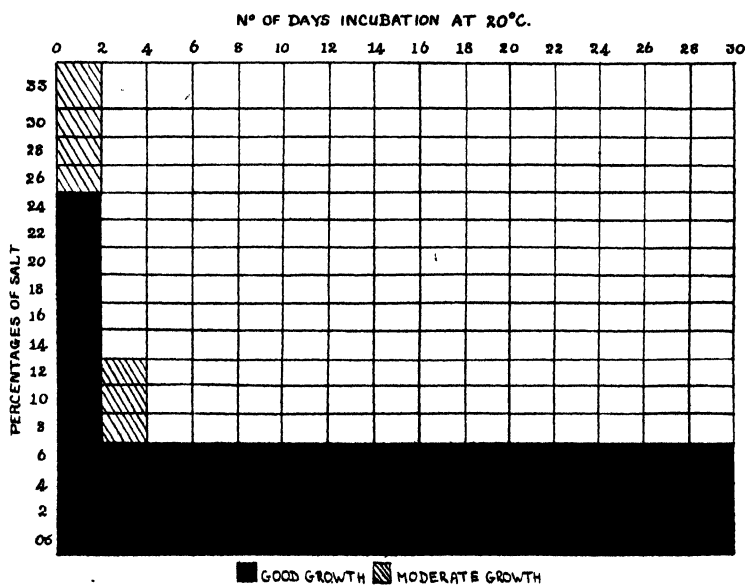
growth, at the other end only one of the non-halophils, *S. lutea*, is capable of survival for more than 10 days. None of the non-halophilic organisms, as is shown in Table I, gave any sign of growth in broth of a salt concentration of more than 8 per cent.

It was concluded, therefore, that the extensive red patches under examination in salted hides were almost certainly due to the growth of genuine halophilic bacteria, and not to the ordinary organisms of dirt and dust, even though these might be present on the hide and be capable of survival in high concentrations of salt.

# Red Colour of Salted Hides, etc.

Fig. 5. *S. lutea*.Fig. 6. *S. aurantiaca*.



*Red Colour of Salted Hides, etc.*Fig. 9. *Actinomyces*.Fig. 10. *B. proteus*.

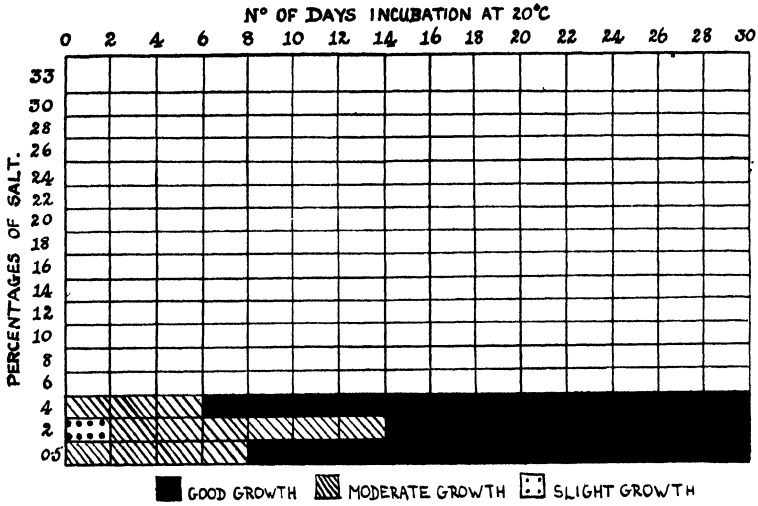


Fig. 11. *B. pyocyaneus*.

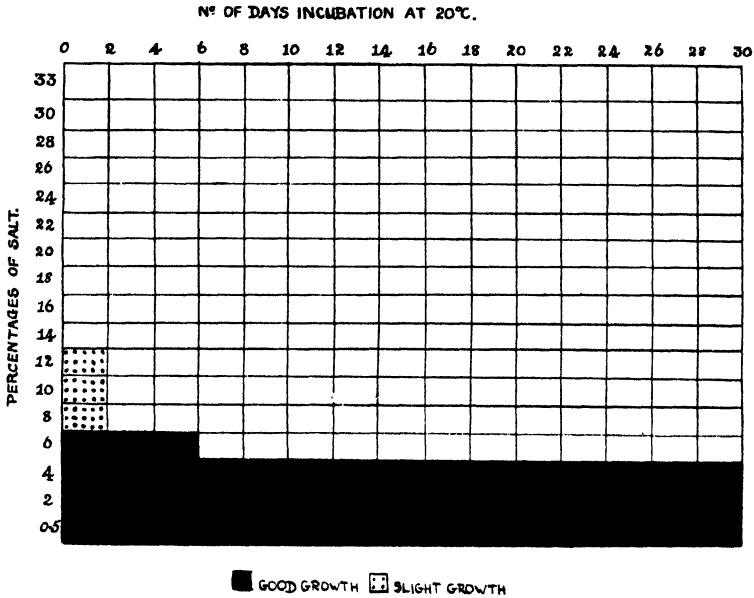


Fig. 12. *B. fluorescens liquefaciens*.

Table I. *The growth of organisms of dirt and putrefaction as shown by the turbidity of the broth cultures after 48 hours.*

Organism	Percentage of sodium chloride in the broth					
	0.5	2.0	4.0	6.0	8.0	10.0-33.0
<i>S. lutea</i> ... ..	Very turbid	Very turbid	Very turbid	Very turbid	Slightly turbid	Clear
<i>S. aurantiaca</i> ... ..	" "	" "	Slightly turbid	Slightly turbid	Clear	"
<i>S. rosca</i> ... ..	" "	" "	" "	" "	" "	"
<i>M. tetragenus</i> ... ..	" "	" "	Very turbid	Very turbid	Slightly turbid	"
<i>B. pyocyaneus</i> ... ..	" "	" "	Slightly turbid	Slightly turbid	" "	"
<i>B. fluorescens liquefaciens</i> ... ..	" "	" "	Very turbid	" "	" "	"
<i>Actinomyces</i> ... ..	Clear	Clear	Clear	Clear	Clear	"
<i>B. proteus</i> ... ..	Very turbid	Very turbid	Very turbid	"	"	"

The results of these experiments confirm what has been noted by other workers (Hill and White (1929), Dubois (1910), Duthoit (1923), Guillemard (1909)) on this subject, *i.e.* that cocci can resist high concentrations of salt much better than bacilli. They indicate also, and most of them were repeated once or twice with the same results, that each type of organism examined reacts in a specific manner to the presence of different salt concentrations in the medium. They thus support the suggestions that have been made by the above-mentioned workers that media of varying salt concentrations might be used as a method of separating organisms in mixed culture and might be of service also in helping in the identification of different kinds of bacteria.

#### CONCLUSIONS.

1. Certain brick-red stains on salted hides are produced by the growth of "halophilic" or salt-loving organisms which come from marine salt used in curing the hide.
2. There are probably many varieties<sup>se</sup> of these organisms, their common characteristic being that they flourish best in high concentrations of salt, and will not grow when the salt concentration falls beneath 6 per cent.
3. The organisms of dirt and putrefaction examined, though certain of them survived for a considerable time at salt concentrations as high as 30 per cent., did not multiply when the salt concentration of the medium was above 8 per cent.
4. The non-halophilic cocci examined survived at high salt concentrations much better than the non-halophilic bacilli.

## REFERENCES.

- BROWNE (1921-22). *Proc. Soc. Exper. Biol. & Med. N.Y.* **19**, 321-2.  
CLAYTON and GIBBS (1927). *The Analyst*, **52**, 395.  
CLOAKE (1923). *Dept. of Sci. & Ind. Res. Food Investigation Board, Special Rept. No. 18*, 23.  
DUBOIS (1910). *C.R. Soc. Biol.* **68**, 26.  
DUTHOIT (1923). *Ibid.* **89**, 548, 550, 553.  
EWART (1887). *6th Ann. Rept. of the Fishery Board for Scotland*, Appendix 204.  
FARLOW (1878). *U.S. Commission of Fish and Fisheries, Commissioner's Report*, 969.  
GUILLEMARD (1909). *C.R. Soc. Biol.* **67**, 538.  
HARRISON and KENNEDY (1922). *Rept. II, Hon. Advisory Council for Sci. and Ind. Res. Canada*.  
HILL and WHITE (1929). *J. Bact.* **18**, 43.  
LAYET (1887). *Bull. U.S. Fish Commission*, **7**, 90.  
LE DANTEC (1891). *Ann. Inst. Pasteur*, **5**, 656.  
LE FÈVRE and ROUND (1919). *J. Bact.* **4**, 177-82.  
MAURIAC (1886). *U.S. Commission of Fish and Fisheries, Commissioner's Report*, 1027.  
STATHER and LIEBSCHER (1929). *Collegium*, 427.  
STURGES and HEIDEMANN (1923). *Abstr. Bact.* **7**, 11.  
—— — (1924). *Ibid.* **8**, 14.

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# EXPERIMENTAL DUST INHALATION IN GUINEA-PIGS.

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(With 6 Text-figures.)

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The experiments forming the basis of this paper were undertaken on behalf of the Health Advisory Committee of the Safety in Mines Research Board, under the general direction of Dr J. S. Haldane, C.H., F.R.S., and with financial assistance from the Board and from the Medical Research Council.

## HISTORICAL.

WHILE the ill-effects produced in man by the inhalation of certain kinds of dust have been recognised since the seventeenth century, it is not until comparatively recently that experiments have been made to determine the nature of the processes leading to the pathological condition. The nature of the inhaled particles was also known to affect the course and result of the pneumoconiosis. In the case of some dusts large amounts were found *post-mortem* in the lungs of individuals who had not suffered from pulmonary disease. When siliceous dusts had been inhaled there was fibrosis of the lungs, very frequently with tuberculosis, which was the immediate cause of death. It was further noted that very small concentrations of silica dust in the inhaled air were sufficient to produce fibrosis and tuberculosis, while very considerably larger amounts of other kinds of dust, including coal dust, had no such effect.

Experiments on the effects of dust on the lung, and the manner in which that organ disposes of the dust, have been conducted along three separate avenues: the earliest method was to expose the animals to a cloud of dust, while later methods were the injection of suspensions of dust intratracheally and intravascularly respectively. It will be obvious that the pulmonary reaction obtained by the first method will most closely approximate to that found in human pneumoconiosis.

Arnold (1885), who was the first worker to dust animals experimentally, used soot, sandstone and emery. He was more concerned with the question of

the elimination of the dusts than with that of the permanent lesions which might be caused by them, and found that much of the dust disappears from the lung in course of time. He described the phagocytic dust cells and their elimination either directly into the bronchi or *via* the lymphatics into the bronchial glands, and considered that the actual phagocytes were derived from the alveolar epithelium.

The next important paper on the pathology of various induced pneumoconioses is that of Beattie (1912), who used coal and various stone dusts. He was one of the first students of the problem to infer that the tendency of a dust to cause pulmonary fibrosis is the main key to its character. He stated that the more irritating the dust the more intense the fibrosis. This statement is only a half-truth, for many harmless dusts cause a marked irritative reaction immediately on their entry into the lung. If, however, a dust only develops its irritative properties after being phagocytosed within the lung, Beattie's statement holds good as a general rule.

Mavrogordato (1918), working on behalf of the Medical Research Council, conducted a series of experiments with guinea-pigs, using various dusts—coal, flue dust, shale, quartzite, precipitated silica and mixed flint and coal. It is unfortunate that in this paper he adopts as his principal criterion of pulmonary damage the formation of plaques of dust cells, and states that the plaques are the only site of fibrosis. This does not agree with the writer's findings. Further, he states that precipitated silica is rapidly eliminated. In the writer's series, while this dust was fairly rapidly removed from the lung, it was found to be very much the most apt to produce fibrosis of all the dusts examined (see p. 109).

To Mavrogordato is due the observation that the colourless dusts administered by him became blackened in the lung. This is also true in the wide range of dusts used by the author: some dusts become much darker than others, but all become pigmented some 6 months after inhalation. In this respect it may be of interest to mention the work of Granel and Hedon (1928), who state that the nucleated cells of the pulmonary epithelium may normally contain melanin. The haemoglobin of dead red blood corpuscles is taken up by cells of the reticulo-endothelial system, passing from the endothelium of the pulmonary capillaries to the alveolar epithelial cells, where it is converted to melanin, which passes away in desquamation of the cells. If this be true, the blackening of intracellular pigment may be due to the deposition of melanin on the particles.

Mavrogordato's most important experiment was the simultaneous administration of coal (notoriously the most easy of dusts for the lung to excrete) with flue dust and with crystalline silica, each of which causes permanent damage when administered alone. He found that the coal in the mixture tends markedly to assist the removal of the harmful ingredient before pulmonary damage has occurred: this if the latter be only in small proportion in the mixture. He also gave intense exposures of dust to guinea-pigs, keeping them for 36 hours in the dusting chamber, using six daily 6-hour exposures. While

only a few animals were used, he noted that "nearly all distinction between the behaviour of the different dusts disappeared" in the animals killed shortly after the experiments. There was much dust in all the lungs, though, in the case of coal and shale, considerable quantities had collected in the connective tissue at the roots of the lung. In animals killed 3 months after the last exposure he found that much more of the coal and shale dusts had been transported to the bronchial glands than was the case with flint and Transvaal dust (quartzite).

This author's conclusions are: Dusts that make mischief are dusts that accumulate. Dusts that are eliminated are dusts that produce a marked initial reaction, with much shedding of epithelium. Dusts that accumulate do not produce so marked an initial reaction, there being much less shedding of epithelium. Dusts that produce an initial reaction tend to carry out with them the more inert.

The same author (Mavrogordato, 1922) continued and amplified the coal-silica mixture experiments, and concluded that silica fixed in the lung is unaffected by subsequent inhalation of coal, but that prior or simultaneous exposure to coal appears to render the lung resistant to the action of silica.

Mavrogordato (1926) recounts the results of a long series of experiments carried out on the Witwatersrand. This paper is concerned with the relations of silicosis and tuberculosis in gold miners, and contains an elaborate summary, from which the following points are taken:

1. There are two types of disabling miners' phthisis: tuberculo-silicosis, where the fibrosis is dominant, and silico-tuberculosis, where the infection is dominant.

2. There are two factors in miners' phthisis: a phthisis-producing dust and the infection.

3. The reticulo-endothelial system is responsible for the phagocytosis of dust and of the tubercle bacillus. The cells of this system, with which he includes the vascular endothelium, though labile in function, may become determined towards their final form (fibrous tissue) under certain stimuli.

4. A phthisis-producing dust probably possesses a slight degree of solubility in the tissue fluids of the body.

5. The effective occupation of the lungs by a phthisis-producing dust may influence the course of infections other than those due to the tubercle bacillus.

6. It may need less accumulation of dust and less duration of exposure to lower resistance than to give rise to a simple clinical silicosis.

7. Coal dust does not appear to influence the resistance of the lungs of guinea-pigs or rats to pulmonary tuberculosis. This conclusion is in conflict with the findings of Wainwright and Nichols (1905), who found that guinea-pigs, after exposure to coal dust and subsequent inoculation of tubercle bacilli, died of tuberculosis but were free from pulmonary lesions. Mavrogordato's explanation is that he probably failed to select a suitable dose of bacteria. It is more likely that the discrepancy was due to the fact that Wainwright and

Nichols administered their tubercle bacilli per tracheam, whereas Mavrogordato injected his intraperitoneally. If the dust cells are derived from the reticulo-endothelial system one would expect the results of the two experiments to be the same, since it is admitted on all hands that the dust cell is responsible for the phagocytosis of the tubercle bacillus. If, on the other hand, the dust cell is derived from the alveolar epithelium, the results of the two experiments are what would be expected. Sewell (1918) studied this problem by the intravenous injection of carmine followed by the intratracheal injection of indian ink, pigeon's blood and other substances. He found that while the carmine particles were ingested by the macrophages of the blood, the carbon particles or foreign blood cells were taken up by phagocytes within the alveoli, which he states are definitely derived from the alveolar epithelium.

Permar (1920) disputes Sewell's findings, and claims that the dust cells are endothelial cells derived from the pulmonary capillaries, and that they invade the alveoli, phagocytose foreign particles, and make their way back through the alveolar walls into the lymphatics.

Westhues (1922) and Carleton (1927), using a technique similar to that of Permar, were unable to confirm his results, and each concludes that the dust cell is of alveolar epithelial origin. The former author washed the cells out of the vascular system of a rabbit and injected indian ink into the trachea. Two fragments of lung were then excised, one fixed immediately and the other placed at body temperature in saline for half-an-hour before fixation. In the former piece no phagocytosis was seen, but in the incubated piece of lung a considerable degree of ingestion of the carbon particles was noted. In supplementary experiments indian ink was injected into the blood vessels after removal of the blood cells. The lung was incubated in saline as before, but on fixation and sectioning no phagocytosis was seen.

Seeman (1925), after the intratracheal injection of iron lactate, demonstrated the presence of iron within cells free in the alveoli, and also attached to the alveolar wall, but after intravenous injection of the same salt was unable to find any iron in the pulmonary capillary endothelium. Further, Seeman showed that the staining reactions of reticulo-endothelial cells and of alveolar epithelial cells to vital dyes are different.

Binet and Champy (1926) and Carleton (1925) found that alveolar epithelial cells proliferate and are highly phagocytic in tissue culture, while endothelial cells are not.

Willson (1928) found that branching of the bronchial tree continues after birth, and new branches are formed both by centripetal and centrifugal processes, so that previous respiratory alveoli become incorporated into non-respiratory bronchioles. The endodermal origin of this highly phagocytic cell can hardly, then, be denied, and Guieyette-Pellissier's statement that the alveolar epithelial cell is so little advanced in differentiation that it can evolve in divers ways according to the circumstances influencing it is probably true.

The weight of evidence is in favour of the alveolar epithelial origin of the

dust cells, and in the course of the present research no phagocytosis by cells definitely not derived from the alveolar epithelium has been seen.

Carleton's publication (1924) is the most detailed study of experimental pneumoconiosis to date. This author investigated the action of mixtures of coal and flint, and found that one part of coal to two parts of flint by measure caused some degree of permanent damage, but no fibrosis, while in a series with the proportions of the dusts reversed the damage was less in degree, though still present. He concludes that "the early phagocytic response initiated by the coal is responsible for the rapid elimination of much of the flint."

The administration of china clay produced permanent lesions, which were also found in a group of animals exposed to half the usual concentration of dust. In a third group, exposed to half the usual amount of dust, but with the 24-hour exposures spread over 6 weeks instead of 14 days, the lesions were less marked, indicating that the lung may in some degree be trained to eliminate such a dust.

Felspar, ground pitcher (as used in the Potteries) and amorphous silica rendered insoluble by heating to 800° C. were all found to cause fibrosis. Flint, however, while causing lesions likely to lead to permanent pulmonary damage, was not found to cause fibrosis. Pure coal was administered to serve as an experimental control to the other dusts used, and was found to be rapidly eliminated without causing any trace of permanent damage. Shale gave results confirming those of Beattie (1912) and Mavrogordato (1918), being removed less rapidly than coal, but causing no permanent damage. To test the theory that coal and shale owe their innocuous properties to their contained organic matter stimulating phagocytosis, a group of animals was dusted with ignited shale, with results similar to those obtained with ordinary shale.

Dried garden earth was found to cause no permanent lesions, though it was but slowly phagocytosed, and ignited earth showed similar results in the early stages, though the experiment was not completed, as most of the animals were carried off by an epidemic of pneumonia. The present writer has found that all the dusts administered by him were rapidly ingested by phagocytes (see p. 104), although the speed of elimination of the dust cells varied considerably with the nature of the dust.

Carleton (1927) describes the effect of haematite and of levigated iron. Haematite was found to initiate a brisk reaction, and to be fairly rapidly removed without causing permanent damage (see p. 110), but iron produced greater inflammatory lesions, and caused a slight degree of fibrosis.

Landis (1925) states that there is no proof that organic dusts are harmful. Much of the appearance of damage from them comes from the large amount of silica and other inorganic dusts in them. Organic dusts at times produce protein intoxications, but they emphatically do not produce pneumoconiosis.

#### METHODS OF EXPERIMENT. .

The experiments were conducted as follows: twelve healthy guinea-pigs, usually not quite full grown, were chosen as a group. The animals were placed,

in cages, in the dusting chamber described by Mavrogordato (1918). Until recently each animal was placed in a jaconet bag, with only its head emerging, before being placed in the cage, this being done to prevent the dust cloud from being lessened by the animals urinating. In the later experiments performed at St Bartholomew's Hospital this has been found unnecessary, however. 140 c.c. of the dust was placed in the trough of the machine, and the animals exposed to the dust cloud for 2 hours. Towards the end of the dusting period the dust flocculated, and flew less well, as pointed out by Mavrogordato. This is in all probability due to its becoming damp. To allow for the dust thus out of circulation, 70 c.c. of dust was added to that already in the machine before the next dusting was commenced. In Group 19, however, where the object was to have the maximum amount of dust in the air, an unmeasured excess of dust was added each day.

Each group received 2 hours' dusting per weekday for a fortnight (24 hours in all) except in the case of Groups 1, 2, 3 and 19. The dust cloud in the machine was thick, but the animals appeared to suffer little, if any, discomfort. That the exposures to the dust were inadequate to affect the health of the experimental animals is shown by the fact that the percentage casualties in the epidemics of pneumonia, which periodically swept through the menagerie, were greater in the control than in the experimental animals. In this connection it may be of interest to mention that casualties have been less frequent since the animals have been kept in the City of London than was the case when they were housed at the Cherwell Laboratories, Oxford, which have definitely rural surroundings.

Two guinea-pigs were killed soon after the last exposure, two others 2 to 6 weeks afterwards, and the remainder at times varying with the indications found in the earlier animals.

In all cases where the survivors were not thinned out by casualties, two animals were killed at the same time, in order to minimise as far as possible the variations due to individual response of the guinea-pigs to dust.

Maitland, Cavan and Detweiler (1921) describe haemorrhagic lesions of the lungs in guinea-pigs, and ascribe them to the mode of killing. According to them, a blow on the head causes many of these lesions, as does also cutting the carotid arteries. They find the lungs most nearly normal in those animals killed by intensive chloroform inhalation. The writer's experience fully bears out their findings, and the animals were as a routine killed with chloroform, death occurring very rapidly. Immediately the animal was dead, the trachea was ligatured at the upper level of the thyroid, and the thoracic viscera removed *in toto*. In surprisingly few cases did autopsy reveal any pathological condition of the abdominal viscera. The haemorrhagic spots on the lung surface caused by the chloroform could almost invariably be distinguished from older ones by their difference in colour in the fresh specimens.

The fixative employed as a routine was mercuric-chloride-formol, experience having shown its superiority over other mixtures for all-round work on the lung. The tissues were immersion-fixed for 24 to 48 hours, and transferred to

70 per cent. alcohol containing iodine to remove the precipitate formed by the mercuric chloride. After 12 hours in this, they were dissected so as to give one section across both lungs at the level of the bifurcation of the trachea, another section being taken across the middle of each lobe. The tissues were dehydrated and cleared in the usual manner, and were freed from air in the vacuum thermostat when in paraffin of 48° C. melting point. Providing that the tissue is kept submerged in the paraffin by means of wire gauze, the reduction of pressure in the thermostat may be taken to the limits of a water-pump. The specimens were embedded in paraffin of 54° C. melting point, and the blocks allowed to set at room temperature, much better results being thus obtained than by cooling them in tap water.

As a routine, 8 $\mu$  sections were studied, but 4 $\mu$ , 25 $\mu$  and 60 $\mu$  sections were occasionally used. Where necessary, serial sections were made.

Delafield's haematoxylin and eosin was the standard staining method used, and sections from all animals were also stained in van Gieson's collagen stain. In cases where a nuclear stain was required to precede this, Heidenhain's iron haematoxylin was used. Curtis' substitute for van Gieson's stain (Ponceau S and picric acid) was found to be unreliable for guinea-pig lungs, as it does not pick out the youngest and finest fibres. Weigert's elastin stain and Ziehl-Neelsen's and Gram's methods were resorted to when necessary.

The staining in van Gieson must be most carefully done in order to get the correct degree of differentiation. This is obtained when the thin subserous layer of collagen in the pleura stands out brilliantly against the smooth muscle with which it is associated.

#### CONTROLS.

Control animals were periodically examined. These were healthy beasts which had for some time lived under the same hutch conditions as the dusted animals. The controls were of all ages, so as to correspond more accurately with the experimental material. There was no constant variation in normal pulmonary condition with age.

The principal points to be mentioned in connection with the normal guinea-pig's lung are:

- (1) There is very little connective tissue present except in the pleura and around the blood vessels and bronchi and their branches. This enables one to detect a very slight fibrosis—one which would pass unnoticed in the human lung.

- (2) Small areas of congestion, with thickening and slight proliferation of the alveolar epithelium, are moderately frequent. Broncho-pneumonia, described by Carleton (1924) in some of his controls, was very seldom observed by the writer. It would appear by the present observations that broncho-pneumonia is rare either in normal or dusted animals.

- (3) A very slight amount of intracellular dust is normally present in the guinea-pig's lung, in the alveolar wall, the pulmonary lymphoid tissue and the bronchial lymph glands. In the case of the controls to this paper, this dust was hardly likely to be carbon, since the hutches were in an exceptionally

smoke- and dust-free atmosphere. It is probable that they were derived from the animals' bedding, and became blackened after deposition in the lung. Although Willis (1922) describes a spontaneous pneumoconiosis in aged normal guinea-pig lungs, no trace of such a condition was observed by the writer. Indeed, in view of the efficiency of the guinea-pig's nasal filter, the occurrence of such a condition would seem to indicate that the animals had been housed under most unsuitable conditions.

(4) Small lymph nodules are fairly common, generally being perivascular or peribronchial, but sometimes occurring in the parenchyma.

(5) As first pointed out by Opie (1904) the lymph nodules and the connective tissue of blood vessels and bronchi are often infiltrated with eosinophils.

#### THE EXPERIMENTAL FINDINGS.

The reaction of the guinea-pig's lung to inhaled dust may be briefly summarised as follows. Immediately after the last inhalation there is usually a fair amount of dust free in the bronchi, and to a less degree in the alveoli. The response of the lung to the first entry of dust is described in the first three groups of animals dealt with below. After the normal exposures, dust cells are numerous, both forming part of the alveolar wall and free in the alveoli and bronchi. Little or no dust is to be found in the pulmonary lymphoid tissue, or in the tracheo-bronchial lymph glands. There is thickening of the alveolar epithelium to a degree varying with the dust used, while there may be epithelial proliferation. In animals killed 3 or 4 weeks after the last exposure there is usually a less marked reaction to the dust than in the first animals. The thickening and proliferation of the epithelium is less general, but is often intense in patches. In such patches there is congestion. To the naked eye the lung may begin to show pigmentation, and there may be one or two haemorrhagic spots. After 3 or 4 months large areas of lung may be normal, except for the presence of dust cells in the alveolar walls. Some degree of thickening is always present, and incipient fibrosis may be found in the areas of most marked epithelial thickening. No matter if the inhaled dust were colourless, it has become blackened by this time, and the naked eye pigmentation of the lung surface has increased. The dust has commenced to enter the lymphatics in fair amount, and the dust cells are found beneath the pleura and in the pulmonary lymphoid tissue. The tendency of dusts to undergo lymphatic elimination varies, and from this time on the lungs of different groups of animals vary in appearance according to the nature of the dust. If this be harmless the number of dust cells in the lung slowly but gradually decreases, while the thickening of the epithelium decreases in intensity and extent. More and more dust cells appear in the lymphoid tissue, which may increase in amount, while others are constantly found on their way up the bronchi. In the case of a "harmful" dust (one which produces permanent pulmonary lesions) the dust appears to undergo removal in only a slight degree, free dust cells being comparatively rare after the first few weeks. Plaque formation may occur, either with or without



**fibrosis**, while fibrosis in areas of marked thickening may exist without the presence of plaques. The fibrosis is very seldom of the nodular variety so generally found in the human lung. In the later stages of a lung containing an irritating dust an eosinophil infiltration is often found; it may be taken as an indication of the likelihood of fibrosis supervening.

The following experiment was performed to discover the nature of the immediate response to a dust known to be one of the "harmless" class. Six animals were exposed to a dense cloud of shale dust for 2 hours. One was killed immediately. Dust was found freely plastered on the epithelium of the trachea and the larger bronchi, while the smaller bronchi contained plugs of mucin with a good deal of entangled dust. Even so early as this, occasional dust cells were found free in the bronchi, while there was very little free dust in the alveoli. A considerable amount of dust had obtained access to the lung. The larger particles were found entangled in mucin in the trachea and bronchi, while the alveoli, especially those immediately beneath the pleura, contained the finest particles, often in large numbers. Many dust cells were found in the alveolar walls, often in process of detachment. The dust cells were most numerous near the pleura, where there was the most dust (see Figs. 1, 2 and 3).

This experiment shows that within a maximum period of 2 hours after the entry of dust into the lung it may not only be phagocytosed, but the phagocytic cells may be detaching themselves from the alveolar walls. No sign of phagocytosis by cells not derived from the alveolar epithelium was seen.

The second animal was killed  $1\frac{1}{2}$  hours after the dusting. There was very little free dust in the trachea, but a moderate amount plastered against the bronchial epithelium. Dust cells were lying free in bronchi and alveoli, the walls of the latter containing many dust cells. There was a very slight amount of dust free in the alveoli. Occasional ciliated columnar cells of the bronchial epithelium contained phagocytosed particles.

There was a marked though patchy thickening of the alveolar epithelium  $4\frac{1}{2}$  hours after the dusting, with some proliferation and a certain amount of congestion. The alveoli were practically free from extracellular dust, but many dust cells were present in the alveolar walls, while some were detaching and yet others were free in the alveoli and bronchi (see Fig. 4).

After  $22\frac{1}{2}$  hours the only free dust seen was in the medium-sized bronchi. Dust cells were actively becoming detached from the alveolar walls. Numbers of dust cells were to be seen free in the alveoli and bronchi.

The last animal, killed  $44\frac{1}{2}$  hours after the dusting, showed no free dust anywhere in the lung. The thickening of the alveolar walls was still to be noticed, while the shedding of dust cells into the alveoli and bronchi continued.

In all the animals the detached dust cells were smaller than those usually met with in animals killed a longer time after the last exposure. They were spherical, with fairly well-staining nuclei. In the animals killed after  $22\frac{1}{2}$ ,  $27\frac{1}{2}$  and  $44\frac{1}{2}$  hours there were many polymorphs in the blood vessels and the



Fig. 1. Animal killed immediately after a single 2-hourly exposure to shale dust. Dust cells and free dust in a bronchus. Dust particles within a ciliated columnar epithelial cell. Sessile dust cell about to detach.

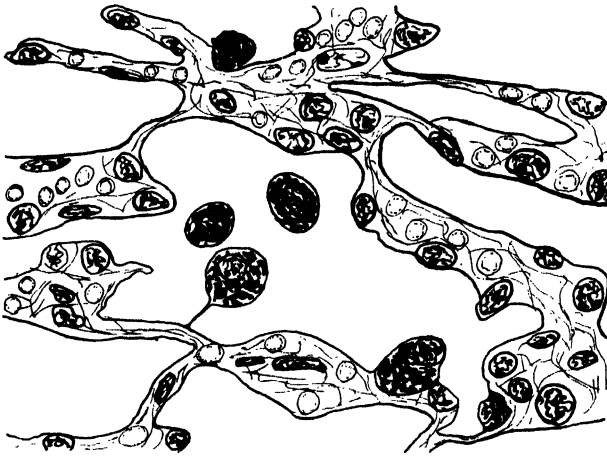


Fig. 2. Animal killed immediately after a single 2-hourly exposure to shale dust. Heavily loaded dust cells free in alveolus, detaching and attached to alveolar wall.

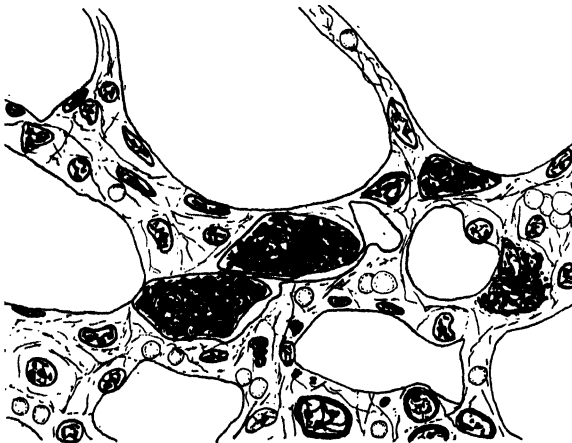


Fig. 3. Animal killed immediately after a single 2-hourly exposure to shale dust. Two very heavily loaded cells filling an alveolus.

parenchyma in those areas where the reaction to the dust was most marked, but no polymorph contained any dust.

A similar experiment was performed to determine the immediate response of the lung to the inhalation of a dust known to cause rapid and permanent lesions. The dust used was pure precipitated silica, as used for Group 5 below.

In the animal killed immediately after the exposure there was a fair amount of dust free in the larger bronchi, and some also in the alveoli. Intracellular dust was found in the bronchial epithelium, while there were numerous dust cells in the alveolar walls and also free in the alveoli and bronchi. It was

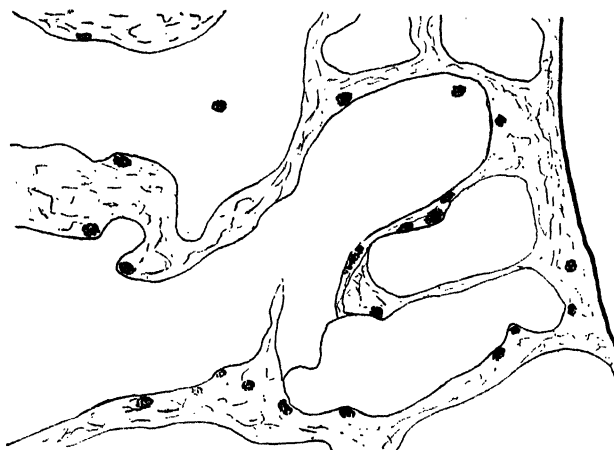


Fig. 4. Animal killed  $4\frac{1}{2}$  hours after a single 2-hourly exposure to shale dust. Low-power view of juxtaleural alveoli (unstained) showing amount and distribution of the dust.

obvious that the entry of the dust had elicited a brisk phagocytic response within the lung, and that it was already being eliminated by the bronchial route.

Two hours after the exposure there was a fair degree of oedema in patches, with some congestion. In the oedematous areas there was some degree of eosinophil infiltration, while, although the total number of dust-containing cells in such areas was not great, a fair number of swollen dust cells were becoming detached from the alveolar wall. Free dust cells were found in the bronchi in fair numbers, together with eosinophils which had certainly in some cases migrated from the adventitia of the bronchi. These cells were usually entangled within mucin plugs, together with a fair amount of free dust. Very little free dust was to be found in the alveoli. In one lobe there was a marked degree of oedema, with considerable migration of eosinophils.

After 6 hours there were numerous eosinophils and fair numbers of dust cells free within the bronchi, with a fair amount of free dust in mucin plugs in the larger bronchi. Patches of marked thickening of the alveolar epithelium were frequent in the apices, and in these areas there was much intracellular dust, while the nuclei of many of the cells were pyknotic. Large numbers of

eosinophils were present in the parenchyma, in the areas of thickening most of them being in a state of degeneration. Many dust cells were found in the alveolar walls, and a fair number free in the alveoli and bronchi. The lesions as a whole were much less marked in the lobes than in the apices.

In the animal killed 24 hours after the exposure there was marked and almost universal thickening of the alveolar epithelium, with a massive eosinophil infiltration not only of the parenchyma but of the adventitia of the bronchi. Numerous eosinophils were seen migrating through the mucosa of the bronchi to become free within the lumen. There was some free dust in the bronchi, but very little in the alveoli. Pyknotic nuclei were common in those areas where there was any great amount of dust. Dust cells were free both in the alveoli and the bronchi.

After 48 hours the condition of the lungs was less severe than at 24 hours, the intensity of the reaction obviously having passed its maximum. No free dust was seen. Dust cells were free in the alveoli and the bronchi. There were patches of marked thickening in the lobes. The dust was by now of a golden colour.

The lung condition was much more nearly normal in the last animal, killed after 96 hours. Fair numbers of dust cells were found free in the bronchi, but not many within the alveoli. The eosinophil infiltration was by now only slight except in the areas of most marked thickening, where there was a good deal of dust. There was a certain amount of bronchitis.

It is obvious from this experiment that there may be very little difference in the phagocytic reaction of the lung to a definitely harmful dust and to a harmless one. The whole of the dust within the lung had been phagocytosed within 48 hours, and the dust-containing cells were being shed into the alveoli within 2 hours after the first moment of exposure. The subsidence of the lung towards a normal condition 96 hours after the exposure, after a marked reaction to the entry of the dust, was most striking, as was also the eosinophil infiltration. This is never seen in the guinea-pig except in the case of an irritating and potentially harmful dust. Another point worthy of notice is the pyknosis of the nuclei in areas where there was much dust. This phenomenon has very seldom been seen in the course of this research.

A group of animals was dusted in the same manner, using flint dust. The reaction of the lung was in all ways similar to that in the previous experiment, but was less intense. No free dust particles were to be found 24 hours after the exposure, but while dust cells were detaching from the alveolar walls, they were doing so in smaller numbers than in the preceding experiments.

*Group 1.* Dust used: equal parts of flint and shale, administered as follows: once weekly for 2 weeks, twice weekly for 2 weeks, and once each alternate day for 12 days, making twelve 2-hourly exposures in all, spaced out over 6 weeks. The aims of this series were twofold: firstly to see if the administration of a rapidly and easily eliminated dust such as shale along with such a dangerous dust as flint would result in the dust cells carrying away the flint along with the

shale, and secondly to see if the lung could be trained to remove a noxious dust more rapidly (and therefore with less eventual damage) than is the case when daily exposures are given. Carleton (1924) used the acclimatisation method of dusting with china clay, and found that with that dust the lung could in some degree be trained to remove the inhaled particles with greater rapidity.

In the earlier animals there was the usual reaction to dust: dust cells in the alveolar walls and free in the alveoli and bronchi, thickening and proliferation of the alveolar epithelium, some congestion, and a gradually increasing amount of dust in the bronchial lymph glands. Indications of the irritating nature of the dust were found in the later animals in the lymphoid infiltration of the blood vessels, and, in the last animal killed (28 weeks after the last exposure), the presence of patches of incipient plaque formation.

The conclusions to be drawn from this series are that the lung may be in some measure trained to cope with a dust, and that shale, administered along with flint, and in at least equal amount, facilitates the removal of the flint and so reduces the liability to pulmonary damage. The greater the proportion of shale in the mixture the less would be the likelihood of damage.

*Group 2.* Dust used: china clay, administered as for Group 1. This group was a repetition of a series examined by Carleton, but using the normal amount of dust in the dusting machine, instead of the reduced amounts used in Carleton's series (1924). The writer's findings differ in some degree from those of that author, but are rather more conclusive. This is no doubt due to the larger amount of dust used in the present series.

There was not a very marked response to the entry of the dust, but some emphysema was noted in an animal killed one month after the last exposure. Incipient plaque formation, which is never caused other than by potentially harmful dusts, was found in two animals killed 14 weeks after the last exposure, incipient patchy fibrosis in two animals at 39 weeks, and definite fibrosis in areas of chronic inflammatory change in animals examined at 60 and 64 weeks respectively.

These facts indicate slow elimination and a slowly developing but progressive damage to the lung. Acclimatisation with this dust in the amounts used is not sufficient to have any appreciable effect on the course and degree of the damage.

In clinical experience, china clay is not a harmful dust, and the lesions found in these experiments may be due to the large amount of dust entering the lungs in a short time. It would appear, however, that the danger of pulmonary lesions must be expected in the case of human beings exposed to heavy concentrations of china clay.

*Group 3.* Dust used: flint, administered as for Group 1. Not much dust entered the lungs of any animal of this series. The early response was not very brisk, but a certain amount of the dust was removed *via* the bronchi. In the later stages the lungs settled down to definite and progressive fibrosis. Plaque formation was not noted in any animal of this series, but incipient fibrosis

was found at 12 weeks, and had become definite at 30 and 54 weeks. The fibrosis was always found in areas of chronic proliferation resulting from the presence of a good deal of dust. Even in the case of flint, one may find areas which contain little dust and are histologically normal, while neighbouring patches containing many dust cells are fibrosed. Acclimatisation was definitely unsuccessful with flint, probably on account of the toxic nature of the silica which is constantly being dissolved away in minute quantities from the ingested particles.

*Group 4.* Dust used: slate. Two hours' exposure each week day for a fortnight, making 24 hours' exposure in all. ("Rapid" method.) In no case did the lungs of animals of this group contain much dust, as it flies badly in the machine. The dust particles are very fine. Slate was tested on account of the anxiety felt as to the liability to pulmonary damage of workers in slate, who are exposed to moderate concentrations of the dust.

The initial reaction was moderately severe, but of the usual type, and, while the dust undergoes progressive bronchial and lymphatic removal, fibrosis was noted at 15 weeks, and was constant in all animals examined thereafter. Since there was little dust in the lungs of any animal, the fact of permanent damage being caused within 15 weeks under these circumstances indicates that slate must be ranked among the definitely harmful dusts, although the decreasing amount of damage in the last animals (31 and 52 weeks) suggests that the harmful action is more transient than is the case with flint. The fact that guinea-pigs of a group often show some slight degree of variation in response to a dust must, however, be kept in mind.

*Group 5.* Dust used: pure precipitated silica, administered by the rapid method, *i.e.* twelve 2-hourly exposures spread over a fortnight. There was not much dust in the lungs of any animal of this group. This dust was used because it seemed difficult to reconcile the conclusion of Mavrogordato (1918), that the dust was eliminated rapidly and without causing damage, with the observations on flint. There was a rapid and marked response to the dust, in the form of thickening and proliferation of the alveolar epithelium, but only 13 days after the last exposure an early but definite fibrosis was noted. With no other dust has such an early onset of fibrosis been described, and the finding is thus at variance with Mavrogordato's observations. The dust certainly tends to disappear from the lung fairly rapidly, but this is only partly due to elimination *via* the bronchi or lymphatics, a considerable amount of it being dissolved. Indeed, it is most probably due to this dissolved silica that the fibrosis occurs so early. The fibrosis was found in patches in the lungs of all animals examined from 13 days to 44 weeks, while in the later specimens there was a good deal of eosinophil infiltration, always a sign in the guinea-pig lung of an irritative process.

Precipitated silica is the most deadly dust investigated, and the utmost care should be taken to avoid its being inhaled in industry.

*Group 6.* Dust used: haematite, obtained from the Cumberland mines by H.M. Mines Department, administered by the rapid method. There was a

considerable amount of dust in the lungs of the animals of this group. There was a marked initial reaction to the entry of the dust, but the thickening and proliferation of the alveolar epithelium gradually diminished in intensity, while the dust was rapidly eliminated, both by the bronchi and the lymphatics. In the last animals examined (34 weeks after the last exposure), while a good deal of dust remained in the lung, all the signs of progressive elimination were present, and there appeared no likelihood of permanent damage being caused. This finding has been borne out by clinical experience. In this series, lymphatic removal played a greater part than was usually the case with a harmless dust, the probable explanation being the unusually large amount of dust which entered the lung (see Group 19, p. 116).

Haematite, inhaled in small amounts, may be regarded as a harmless dust, but in large amounts the irritation produced might, apparently, cause definite pulmonary symptoms.

*Group 7.* Dust used: carborundum, administered by the rapid method. This dust was chosen as an example of an almost completely insoluble material. In the first animals killed, 4 hours after the last exposure, there was very little response to the presence of the dust. Although the majority of the particles had been ingested by dust cells, there was little thickening of the alveolar epithelium, and only a slight amount of proliferation. At 24 days, however, the reaction was intense in patches, where there was occasional plaque formation and incipient fibrosis. At 19 weeks the removal of the dust, which had hitherto been very slow, was much hastened, while at 52 weeks large amounts of dust had been carried to the pulmonary and bronchial lymphoid tissue. The fibrosis never became more than very slight.

Carborundum is an important dust as illustrating the influence of the dissolved portion of an inhaled dust on the rate of elimination of the dust-containing phagocytes. In this case, the dust being so slightly soluble, elimination was very slow in onset, the particles acting at first as inert foreign bodies, but at 4 months a slight but sufficient amount of solute has perhaps been liberated materially to hasten the removal of the phagocytes.

This dust is one which would in human experience probably cause little damage if inhaled only in small amounts, but would undoubtedly be dangerous if much were present in the air.

*Groups 8, 8 a.* Dust used: wood charcoal, administered by the rapid method. Since the remaining animals of Group 8 died 4 months after the last exposure, Group 8 a was dusted to study the later stages of the pulmonary reaction.

While coal is the least harmful of all dusts, and although it has been so much investigated, the reason for its harmlessness is not at all clear. It has been suggested that the soluble calcium salts present stimulate phagocytosis and elimination, but this is no adequate explanation, since calcium carbonate itself is a harmful dust (see Group 10). It was thought that the carbon in coal might be the cause of the rapid elimination and the freedom from damage, and therefore wood charcoal was chosen as a reasonably pure form of carbon which yet

contained mineral salts which might be thought to stimulate the elimination of the dust.

Under the experimental conditions much dust entered the lungs in both groups. In the first animals there was a surprisingly slow response to the presence of the dust, free particles being found both in the alveoli and bronchi 24 hours after the last exposure. This is a somewhat uncommon condition in the course of these experiments, and may usually be taken as an indication that the dust functions rather as an inert foreign body than by virtue of its solutes. A further suggestion as to the potentially harmful character of the dust was given by the lymphocyte infiltration ("cuffing") of the blood vessels in both the 24 hours' and 26 days' specimens. At 12 weeks there was a drift of the dust towards the pleura. The drift had become marked at 17 weeks, while in both cases an incipient patchy fibrosis was noted. Neither animal killed at 26 weeks showed any fibrosis, and the general pulmonary condition had much improved, though an eosinophil infiltration of the parenchyma indicates that there was considerable chronic irritation. These specimens showed very well that the dust was largely removed by the lymphatic route. In an animal killed 53 weeks after the last exposure there was some plaque formation, and a very slight patchy fibrosis.

*Group 9.* As it was thought that the permanent damage inflicted on the lung by wood charcoal in the last group might have been due to the very large amount of dust which entered the lungs, a batch of animals was exposed to the same dust for 12 days, but only one-third of the usual amount of dust was put in the machine for each dusting. Since wood charcoal easily forms a dense cloud, however, a considerable amount of dust entered the lungs of the animals of this group also.

The early stages showed the same picture as in Group 8, with the dust being largely removed by the lymphatic route. Animals killed at 19 and 49 weeks showed a slight patchy fibrosis in areas of chronic thickening. The damage caused was not very marked, and since the animals were exposed to a concentration of dust unlikely to be met with industrially except under the most unusual circumstances, wood charcoal should be regarded as a border-line dust, in that it would, if inhaled in large amount, be very likely to cause permanent damage, while smaller doses would be comparatively harmless.

*Group 10.* Dust used: precipitated chalk, administered by the rapid method. This dust was used to test the effect of a fairly soluble dust, in order that comparison might be made with the soluble silica series. At this stage of the research a theory as to the causation of damage had been formed, and the majority of the later experiments were carried out to test it. It was thought that the degree of solubility of inhaled substances materially affects both the degree of damage caused in the case of harmful dusts, where the solute is noxious to the tissues, and the rate of elimination of the more harmless ones.

In Group 10 there was a widespread though not marked early reaction, which at 9 weeks had proceeded to a patchy thickening and proliferation of



the alveolar epithelium, sometimes intense, and there was incipient fibrosis in some of these areas in both animals. At 28 weeks the thickening and proliferation persisted, and was often intense in degree. Fibrosis was slight but definite, although there was much normal tissue, and the dust particles were being eliminated by the bronchial route. At 55 weeks there was more normal lung, and the dust was still being passed out of the lung both *via* lymphatics and bronchi, but nevertheless there was a slight patchy fibrosis in both animals, while eosinophil infiltration of areas of thickening showed the presence of an irritant.

Clearly this dust is to be regarded as a potentially harmful one, although it is removed to a considerable degree by the lung. Precipitated chalk is fairly soluble, and it would seem logical to attribute at least part of the damage to the solute's acting as an irritant.

*Group 11.* Aluminium hydroxide, administered by the rapid method. This group was dusted as an additional experiment on solubility factors, and also with a view to Group 14, which is a logical advance on the evidence obtained.

There was not a very marked early reaction, but at 9 weeks the response was much more marked (cf. Group 10). There was an incipient patchy fibrosis in one animal. At 30 weeks there was a considerable increase in the rate of removal, both lymphatic and bronchial, but both animals showed fibrosis. At 72 weeks the condition was more serious, plaques being present as well as fibrosis, and the dust was largely to be found in the interalveolar septa, while at 106 weeks the lesions were yet more marked.

Aluminium hydroxide is obviously a progressively harmful dust, causing only a slight early response, which later becomes more brisk and then falls off again to a largely lymphatic removal.

*Group 12.* Dust used: calcspar, administered by the rapid method. This substance is crystalline calcium carbonate, and, being crystalline, is not so soluble as the amorphous precipitated chalk. It might, therefore, be expected to offer some comparison with the latter.

More dust was found in the lungs of this series than of Group 10, which would tend to intensify the degree of damage. There was a brisk reaction to the entry of the dust, and at 48 hours a good number of dust cells were found free in alveoli and bronchi. At 10 weeks the reaction was still lively, and dust cells were seen migrating from the peribronchial lymphatics to become free within the lumen of the bronchus. It is of interest to note that the dust was blackened by now. At 20 weeks there was some setback, and a very slight fibrosis was found in both animals. At 66 weeks, however, there was a largely normal lung, with no fibrosis, and the dust undergoing drift towards the pleura. At 74 weeks there was widespread but slight thickening and proliferation of the alveolar epithelium, patches of marked plaque formation, and a slight patchy fibrosis.

Dust cells were free in the bronchi and alveoli in all the animals of this group, but the pleural drift and (in the last animal) plaque formation indicate that lymphatic removal also plays a not unimportant part in the elimination

of the dust. This dust is, therefore, to be regarded with some suspicion, as are all dusts tending to cause plaque formation.

The apparently contradictory states of animals at 20, 66 and 74 weeks is indicative that the dust is a border-line one, and that the variation in response depends on individual susceptibility.

Correlating the results of Groups 10 and 12, it is seen that the precipitated chalk produces definitely more harmful results, especially when it is remembered that Group 12 received much more dust than Group 10. This was not due to variations in dusting technique, but to the fact that chalk becomes more easily damped in the dusting chamber and, therefore, flies less well than calcspar. Since the two substances are identical except for their solubilities, the solute can be the only explanation of the variation in reaction of the lung. It is suggested that on the inhalation and phagocytosis (which follows very rapidly) of a dust a certain amount of solute is formed, which stimulates not only the phagocyte but the neighbouring tissues. If the stimulus be a mild one, the response is the detaching of a cell and its passage into alveolus or lymphatic, but if the solute be a cell poison such as silica, not only may the phagocyte be killed by the toxin but the solute diffuses out into the neighbouring tissues and affects them. It would appear to the writer that only on this hypothesis can be explained the onset of fibrosis in an area where there are very few dust cells and very little dust.

*Group 13.* Dust used: emery, administered by the rapid method. The variation in solubility between aluminium hydroxide and emery is very much greater than that between chalk and calcspar, emery being almost completely insoluble. Emery consists of crystalline corundum ( $\text{Al}_2\text{O}_3$ ) associated with small and varying amounts of magnetite, tourmaline, garnet, etc. (Geikie, *Text-book of Geology*, London).

The initial reaction was fairly marked, and dust cells were free in alveoli and bronchi. At 8 weeks the usual lesions were found, but in no marked degree, while at 31 weeks there was some plaque formation in one animal and incipient patchy fibrosis in both. In a 106-weeks' casualty there was a slight patchy juxta-pleural fibrosis.

The indications are that emery behaves as do all approximately insoluble dusts, being largely (after the initial few weeks) removed *via* the lymphatics, with the tendency to damage that this method of removal implies. The fibrosis which is caused by such dusts is the result of inert foreign-body irritation, the distribution of fibrosis in the 106-weeks' specimen being indicative of this.

The soluble hydrated alumina is a more dangerous dust than the insoluble anhydrous crystalline emery, and, as in the case of chalk and calcspar, the causative factor must be the solute.

Irving Clark (1929) states that continued inhalation of the dust of artificial abrasives, consisting of emery or carborundum, did not produce *disabling* pulmonary lesions in men exposed for from 10 to 41 years.

*Group 14.* Dust used: mixed aluminium hydroxide and precipitated silica.

In this experiment the dusts were mixed in the proportions in which they occur in shale. Shale (*U.S. Geol. Survey Bull.*) contains on an average 59 per cent.  $\text{SiO}_2$ , 15 per cent.  $\text{Al}_2\text{O}_3$ , 4 per cent.  $\text{Fe}_2\text{O}_3$ , 2.5 per cent.  $\text{FeO}$  and 1 to 3 per cent. each of  $\text{CaO}$ ,  $\text{Mg}$ ,  $\text{K}$  and  $\text{Na}$ , with traces of other metals.

There was a lively reaction to the mixture and the dust was progressively removed. At 24 weeks there was much lung normal except for the presence of dust, and no permanent lesions (nor any indication of their supervening) were found in any animal.

The mixture was composed of the actual dusts used in Groups 5 and 11, which caused fibrosis in 2 and 9 weeks respectively. The conception underlying the experiment was that the dusts when phagocytosed would produce their solutes within the cell, and that these would combine to form silicate. Since the mixture proved harmless in practice it would appear that this happened and, therefore, that not only are many natural silicates harmless if at all soluble, but that similar compounds formed within the body are themselves harmless. The liberation of a solute into the cytoplasm of a phagocyte, and therefore by diffusion into the surrounding tissues, renders the phagocyte obnoxious to those tissues and so causes it to be cast off. Some solutes may be toxic and yet not provide an adequate stimulus for the casting off of the cell, which is thus enabled to diffuse more of the toxic substance. Silica is a type of dust whose solute is toxic, while many natural silicates are not. Here it is not possible to speak more definitely about silicates, as they are very varied in chemical constitution, and this may be the explanation of the harmful nature of china clay, where the silica is present as a disilicate, and of felspar, where it is a trisilicate, while the harmless shale largely consists of monosilicate.

*Group 15.* Dust used: light magnesium carbonate, administered by the rapid method. This dust was used in view partly of its solubility and partly since naturally occurring magnesium silicates are common, and a series similar to Groups 5, 11 and 15 was thought likely to furnish a useful control to the results obtained from those groups.

Although very little dust was present in the lungs of this group, there was a very marked initial reaction to its inhalation. The violence of the reaction continued, and the dust was predominantly eliminated by the bronchial route. Sub-acute irritation was none the less shown by the presence of eosinophil infiltration at 4 weeks, and at 16 weeks fibrosis was noted. At 31 weeks much of the dust had been removed, yet very few dust cells were found in the tracheo-bronchial lymph glands, indicating that the majority of the dust cells had been removed from the lung through the bronchi. There was still, however, a good deal of thickening and proliferation, and in these areas a slight fibrosis was to be found.

The indications are that here is a dust which, save for its somewhat undue solubility, would be rapidly eliminated by the lung without the intervention in any marked degree of lymphatic removal, but the dissolved substance is of such a nature as to cause a fibrosis.

The dust, therefore, if inhaled in quantity by man, would cause permanent pulmonary damage, though whether tuberculosis would supervene upon such damage is a moot point.

*Group 16.* Dust used: one part of precipitated silica to three parts of light magnesium carbonate by weight, administered by the rapid method. These proportions are those in which the constituents occur in talc. The majority of the dusted animals died during an epidemic of pneumonia, but 24-hour specimens showed a marked general reaction, although not much dust was present. Animals killed as long as 54 weeks after the last exposure had a large amount of normal lung, with some eosinophil infiltration of the parenchyma, but no fibrosis. While eosinophil infiltration in 3- or 4-month specimens is generally an indication of a later fibrosis it may be taken when occurring as much as a year after the original exposure to indicate a degree of irritation short of that necessary to produce a fibrosis. The fact that no fibrosis was found after 54 weeks is indicative that the mixture is, to say the least, much less harmful than is the case with either constituent alone.

*Group 17.* Dust used: talc, administered by the rapid method. Talc is a naturally occurring magnesium silicate. In the case of the natural dust, the irritation and course of the pulmonary reaction are somewhat different from those with the mixture of silica and magnesium carbonate. The early reaction is not so marked, and initial phagocytosis is not so ready, but the later specimens show that the solute merely stimulates elimination of the dust cells, and in no way acts as a toxic agent, even although lymphatic removal plays a not inconsiderable part in the removal of the dust. Even at 60 weeks, both bronchial and lymphatic removal are occurring moderately briskly, and with no signs of pulmonary damage. This degree of lymphatic removal without pulmonary damage has not been met with in any other dusts investigated, and must be ascribed to the very low degree of toxicity of the substances dissolved from talc, which yet are sufficiently obnoxious to the neighbouring tissues to cause the removal of the cells containing the particles.

*Group 18.* Dust used: "colloidal" coal, administered by the rapid method. This experiment was performed to determine the effect of very finely divided particles. The coal was ground wet in a colloid mill, but the particles were not as fine as might be desired, on account of the leaf-like structure of coal, so that the particles were largely in the form of very thin plates. Coal dust is generally in this form, which disposes of the theory that dusts cause damage by the irregularity of their particles. Nothing sharper than a thin plate with irregular edges can be imagined, yet coal is the least harmful of dusts.

A large amount of dust entered the lungs, and plugs of dust cells were found in the bronchi at  $1\frac{1}{2}$  hours. The lung was largely unaffected except for some thickening and proliferation of the alveolar epithelium. At 5 weeks a good deal of dust was found in the bronchial glands, and much more at 12 weeks. No permanent damage appeared likely to be caused, even though there was a parietal drift of the dust. This is probably because the small amount of sub-

stance dissolved from coal is non-toxic, and yet briskly stimulates elimination. The parietal drift and the removal of dust to the bronchial glands indicate that the lymphatic route is employed as well as the bronchial, but this is only likely to lead to trouble when the dust is inert or toxic, or is present in the lung in large amount (see Group 19). In this respect the writer's investigation of the pit pony's lung (Haynes, 1926) showed that dust aggregations are always found in the perivascular, peribronchial and subpleural lymphatics, and in any lymph nodes in the parenchyma, although the bronchi always contain shed dust cells. In some ponies the writer found massive lymphatic aggregations, yet in no case was fibrosis found.

In the present case coal dust, as finely divided as possible, and in fairly heavy concentration, is not harmful.

*Group 19.* In order to examine the effect of such finely-divided coal dust when inhaled in massive amounts, a group of animals was exposed to "colloidal" coal dust for twenty-four 2-hourly periods extending over a month, double the usual quantity of dust being placed in the machine for each exposure. The experiment is really an enquiry into the effect of the entry of immense numbers of particles into the lung, with the consequent shock to the pulmonary tissues.

At 24 hours (*i.e.* 29 days after the *first* exposure) enormous amounts of dust were present, and in so little time a well-marked parietal drift was found. The general reaction of the lung was intense, but the dust was being dealt with in an orderly and normal manner, no lesion other than the inevitable thickening and proliferation of the alveolar epithelium being found. At 39 days there was an improvement in the pulmonary condition, and the dust was being progressively removed. Mechanical interference with the functioning of the lung, however, was indicated by some emphysema in one animal. The other animal, killed 39 days after the last exposure, had a good deal of broncho-pneumonia with red hepatisation, but in view of the rarity of this condition in the experimental animals it is probable that this was not directly due to the dust, though the interference with lymph drainage due to the intralymphatic dust cell masses probably lowered the resistance of the lung to what was in all likelihood a bacterial infection. No bacteria were seen in the sections, however, either in this animal or in casualties dying of pneumonia.

At 26 weeks intralymphatic aggregations of dust were macroscopically easily visible, and the bronchial lymph glands were black with dust. A fair amount was also found in the spleen. Dust cells were still being actively shed from the alveoli, but in places where the dust was present in large amount the parenchyma was infiltrated with eosinophils.

The condition of the last specimen is rather disturbing, as eosinophil infiltration must be taken as indicative of a chronic sub-acute irritation when found not too long after the last exposure, as has been already mentioned. This irritation may in time provoke a fibrosis.

The important deductions to be drawn from this experiment are that the

lung responds nobly to massive exposures to a harmless dust, but with the passage of time the large entry of dust into the pulmonary lymphatic system seriously deranges that system by mechanical occlusion, with a consequent lowering of local resistance to infection. Further, this occluded dust holds back other dust cells, which must therefore lie in the alveolar wall for bronchial elimination, which is harmless, or pass into the peribronchovascular lymphatics, where their continued presence acts as an irritant and may in time lead to a fibrosis.

Unfortunately the later animals of this group were wiped out in a serious epidemic which killed 85 per cent. of the animals in the menagerie, and a definite statement as to the very late effects of such massive exposures to coal dust cannot be made. Sufficient evidence has been gathered, however, to state that any dust, no matter how harmless it may be in small doses, is dangerous to health if inhaled in large quantity. Definite and serious impairment of pulmonary function occurs fairly rapidly. These conclusions are of the utmost importance in view of modern mechanical methods of coal-getting, in which unless precautions are taken to diminish the amount of dust in the air (*e.g.* by wet-working) the miner is exposed to very much heavier concentrations of dust than is the case where the older method of mining obtains.

In this connection it is of importance to mention the work of Collis and Gilchrist (1928), who state that clinical observation by X-rays shows that, after many years of work, the lungs of coal trimmers are not normal, and exhibit signs similar to those widely regarded as characteristic of silicotic fibrosis. In a personal communication, Haldane informs me that similar X-ray appearances have recently been found, in the course of investigations under the direction of Prof. Cummins, in numbers of South Wales colliers. In the case of colliers the matter is somewhat complicated, since such workers always inhale stone dust along with coal dust. It is occasionally the case that colliers have to work through stone containing a large proportion of free silica in order to reach the coal-seam. Middleton (1929) states that in such cases, once enough silica has been inhaled to produce fibrosis, the subsequent inhalation of coal dust into a lung in which the lymph drainage has been disorganised has the effect of increasing the fibrosis and producing disability. It must be borne in mind, however, that, as the preceding experiment demonstrates, disorganisation of the lymph drainage may be produced by the inhalation of coal dust alone, or, indeed, by the inhalation of any dust in massive amounts. Middleton (*loc. cit.*) states that coal dust may (without any evident exposure to silica) produce a condition in the lungs which radiographically resembles the appearances produced by silicosis. Here again the above experiment shows that such appearances are only likely to be produced in the case of exposure to very heavy concentrations of dust. There is no evidence that such a pure anthracotic condition carries with it any increased liability to tuberculosis, although naturally the pulmonary efficiency of the affected individual will be decreased.

*Group 20.* Dust used: shale, administered by the massive method, as follows. An excess of dust was placed in the dusting machine, so that the

densest possible cloud was obtained. Two hours' exposures were given on each of the first 3 days and 4 hours' exposures on the remaining 9 weekdays, so that it might be imagined that the animals would inhale almost as much dust in the 12-day exposures as did the animals in the 24-day exposures of the last experiment. In practice this was found not to be so, since not nearly so much dust was found in the lungs of the majority of the animals of this group as in those of Group 19. This may be of importance when considering human experience, where the exposures are seldom less than 4 hours' duration.

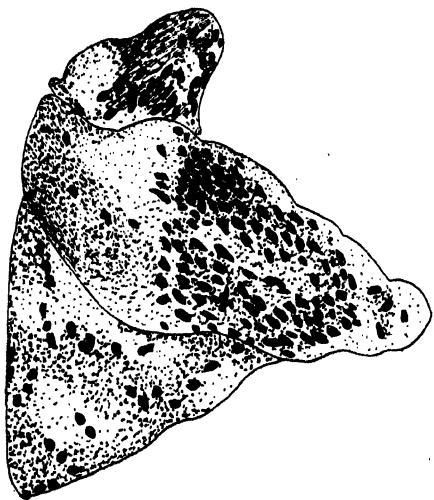


Fig. 5. Lateral aspect of right lung of animal killed 12 months after massive exposure to shale dust.

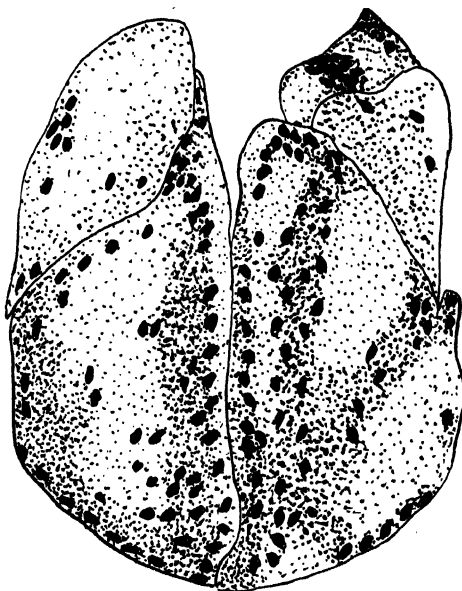


Fig. 6. Dorsal aspect of lungs of animal killed 12 months after massive exposure to shale dust.

In the first animals, killed 4 days after the last exposure, there was a good deal of dust present, with general thickening of the alveolar epithelium, and patchy intense thickening and proliferation. Comparatively few free dust cells were present, but in one animal there were peribronchiolar dust aggregations.

At 11 weeks there was little sign of aggregation of the dust, but in the azygos lobe of one animal there was some giant cell formation. The reaction to the dust was general, but only marked in patches. In one animal there was much normal lung, but a comparatively small amount of dust.

At 8 months there was much approximately normal lung, but many free dust cells were present, although the dust was undergoing lymphoid aggregation, particularly in the lobes.

After 10 months there was a fair amount of dust present, with numbers of dust cells free in the bronchi, while some dust cells were seen passing through the bronchial wall from the lymphatics. Plaque formation was present, especially subpleurally. Dust was present in considerable amount in these areas, and

also in the subpleural, peribronchial and perivascular lymphatics in the apices. A good deal of dust was present in the tracheo-bronchial lymph glands in both animals.

In the last animals, killed 65 weeks after the last exposure, there was a good deal of dust in the lung, but much of the parenchyma was approximately normal. There were numbers of subpleural plaques, however, and a general lymphatic distribution of the dust. There was an incipient fibrosis in one or two areas of plaque formation. The naked-eye appearances of the lungs at 12 months are indicated in Figs. 5 and 6.

The dust used in this experiment was the same as that used for stone-dusting in collieries. It would appear that even such a comparatively harmless dust as shale cannot be inhaled in large amounts with impunity, and the results confirm the deductions made from Group 19 above.

#### RESULTS OF EXPERIMENTS.

The guinea-pig is the animal which has been most widely used for experimental pneumoconiosis, and it has been objected by some critics that findings in these animals cannot logically be applied to human experience in the naturally occurring condition, on account of the considerable difference in structure between the lungs of experimental animals and of man.

The same objection has been raised against all forms of animal experiment, with as little justification. It is sufficient to know the variation in response between the experimental animal and man to normal stimuli, and with that criterion to be able to estimate very accurately the condition in man comparable with the effects produced in the experimental animal.

Certain dusts produce certain effects in guinea-pigs. These dusts have well-known effects on man, and it may definitely be taken that, notwithstanding the comparatively very delicate structure of the lung in guinea-pigs and the consequently apparently slight permanent effects caused by dusts dangerous to man, the unvarying response of the guinea-pig's lung to dangerous dusts renders this animal a very suitable subject for pneumoconiosis experiments. The fibrosis obtained in guinea-pigs is, under the circumstances of these experiments, of the diffuse variety, while that usually seen in human fibrosis is of the nodular variety. Experiments are now proceeding in an attempt to produce a nodular fibrosis by means of dust-inhalation in guinea-pigs, when the response of the lung to bacterial infection will, it is hoped, offer a better comparison with that of man.

These experiments have shown that fibrosis may be caused by many apparently innocent dusts, that is, dusts whose inhalation in man is not followed by increased mortality from pulmonary disease. What is lacking in the experiments is any correlation between fibrosis and liability to tuberculosis. Since, in human experience, some of these dusts do not appear to predispose to tuberculosis, it would not seem that there is any necessary relation between fibrosis and a liability to tuberculosis. It must be borne in mind, however, that



a fibrosed lung must reduce the efficiency of the worker, and that, whether predisposing to tuberculosis or not, the inhalation in considerable amount of any dust tending to produce fibrosis should be avoided.

As the weight of clinical evidence accumulates, silica-containing dusts stand first on the indictment of causation of pulmonary tuberculosis. According to the work of Kettle this is due to the fact that silica is not only a cell poison, but also forms a medium favourable not only to the survival but to the multiplication of the tubercle bacillus. Policard, Doubrow and Boucharlat (1929) found that the addition to tissue cultures of embryonic chick lung of suspensions in Tyrode's solution of crystalline silica obtained from mine rock did not interfere with the development of the culture. The silica was phagocytosed, however, and had a slow toxic action on the engulfing cells. It appears likely, therefore, that the hypothesis of Heffernan (1929) that silica exerts its deleterious action by means of its adsorption on the surface of cells is not well founded. Again, Middleton (1929) mentions that silicosis, while insidious in onset, may proceed after cessation of inhalation if the dose were heavy. Occasionally, very rapid forms of silicosis are found, particularly among those employed in the manufacture of dry soap powders and cleansers, where alkali is present in the dust along with silica.

As mentioned on p. 114, silicates in general are not particularly harmful, but Badham (1927) reports a rapidly fatal case of silicatosis resulting from the inhalation of orthoclase (potash felspar, a trisilicate) dust containing no free silica. He further states that a fine diffuse fibrosis is found in Broken Hill miners inhaling dust containing less than 10 per cent. of quartz but much iron and manganese silicates. The classification of silicates according to their pulmonary effect would be of considerable value in the elucidation of the problem of silicosis.

While, on account of the delicacy of the guinea-pig's lung, it is easy to recognise a slight degree of damage, it is necessary for experimental purposes to expose the animals to concentrations of dust much heavier than those usually met with in industrial experience. This is in order that a sufficient amount may be introduced into the lung. That the amount inhaled was not sufficient to interfere with the health of the dusted animals is shown by the facts that no animal died in the dusting chamber, and that the dusted animals suffered rather less mortality during the pneumonia epidemics which swept through the menagerie than did control animals.

It must be borne in mind, however, that the "onset-mass" of the dust was considerable, and might tend to produce lesions which would not occur were the animal more gradually acclimatised to the dust. In this respect the findings in Group 19 are of interest.

In most of the animals killed immediately after the last exposure to the dust, occasional ciliated columnar cells of the bronchi contained ingested particles. To the writer's knowledge, phagocytosis by such highly specialised cells as functional ciliated columnar epithelium has not previously been described.

Reference has been made (p. 103) to the occurrence of eosinophils in the guinea-pig's lung. When the dust inhaled was such as to cause a chronic irritation short of the degree necessary to produce fibrosis, there was generally found a considerable increase in the number of eosinophils present in the parenchyma. In some cases the eosinophils were so concentrated in a chronically proliferated area that the tissue was solidly infiltrated with them.

In many animals it was found that there was a considerable increase in the number and size of the lymph nodules in the lung. This increase can be taken (on the results of this work) to be indicative of the irritation caused to the lung and its lymph drainage by the dust.

Plaque formation is a criterion first adopted by Mavrogordato as an indication of permanent damage to the lung. In the writer's opinion, the term is misleading, as the so-called plaques, when studied in thick or serial sections, consist of more or less solid aggregations of the cells whose individual nature is so well described by Carleton (1924, p. 445).

The writer, while agreeing that plaque formation indicates some degree of permanent damage, would suggest that the appearance of such structures may reasonably be ascribed to the fact that the atria have become obstructed either by the accumulation of dust cells within their surrounding lymphatics or by direct obstruction by engorged dust cells, so that other dust cells becoming free are unable to pass from their alveoli, and therefore remain free within them. The picture eventually produced is not unlike that of a small area of grey hepatisation, except that the cells filling the alveoli are dust cells and not polymorphs. As the dust cells degenerate and disintegrate their contained particles are liberated, to be phagocytosed again, with further irritation to the alveolar wall. Unless the obstruction to bronchiolar elimination be removed this continued irritation will result in fibrosis, while if free passage be allowed to the plaque cells—which are merely ordinary dust cells—they will pass out *via* the bronchi and cause no fibrosis.

This explanation appears to be the only one which will cover the facts of plaque formation occurring in some animals of a group and not in the later ones, even allowing for the variation in individual response.

It may be mentioned that when an insoluble dust has been inhaled a large part of it is present in the lung after the lapse of over a year. When inhalation is continuous there is a tendency with a benign dust such as coal or shale for a balance to be reached, and the daily removal of dust to become equal to the intake (Haynes, 1926).

#### SUMMARY.

The following dusts produce a fibrosis in the guinea-pig's lung, and are therefore to be classed as dusts whose inhalation in industry would be attended by risks of pneumoconiosis. The most deadly of all dusts examined was precipitated silica. Less dangerous, but all producing fibrosis, were the following, arranged in order of decreasing toxicity: flint, slate, aluminium hydroxide, pre-

cipitated chalk, magnesium carbonate and carborundum. In the concentrations used in the experiments calcspar and emery were border-line dusts, indicating that their inhalation in any considerable quantity would cause fibrosis. Wood charcoal inhaled in large amount produces a slight fibrosis, and must, therefore, be placed on the "dangerous" list. Colloidal coal, when inhaled in massive amounts, is potentially dangerous, while shale under similar conditions is rather more dangerous.

Haematite, talc, and molecular mixtures of soluble silica with aluminium hydroxide and magnesium carbonate respectively were not found to cause any permanent lesions in the lung.

The deductions to be drawn from this work are:

1. All inhaled particles are rapidly ingested by certain individual cells belonging to the alveolar epithelium.

2. These cells (dust cells or phagocytes) remain in the lung parenchyma until they have ingested an amount of dust constituting the cell's saturation load. This load varies with different dusts.

3. A cell having attained its saturation load becomes sooner or later detached from the alveolar wall and either migrates into the lymphatics or becomes free in the alveolus. In the former case it passes into the pulmonary lymphoid tissue and thence to the bronchial lymph glands. In the latter case it passes up the bronchial tree to be either coughed out or swallowed.

4. Dust cells which speedily leave the alveolar wall are principally eliminated by the bronchi.

5. In the case of a dust cell being eliminated from the lung *via* the lymphatics, it may be arrested in the peritrial lymphatics on account of its bulk. The dam thus produced offers obstruction to the passage of other dust cells shed into the alveoli. Groups of free dust cells in the obstructed alveoli form plaques, which degenerate and liberate their dust. This is again ingested, and the irritation caused by such a process may lead to fibrosis.

6. The continued presence of dust-laden cells in the lymphatics may set up a foreign body irritation, with resulting fibrosis.

7. Most inhaled particles contain soluble matter to at least a very small extent. The solute may be either harmlessly active or toxic. If the former, the cell is stimulated to detach itself from the alveolar wall, and so remove the dust. If the latter, the solute effects the viability of the phagocyte, which becomes less able to detach itself. At the same time the solute diffuses into the neighbouring tissues, with irritation to them, and consequent fibrosis.

8. The more soluble form of a substance causes greater pulmonary damage than the less soluble. The solute, therefore, plays a large part in the determination of damage.

9. While many dusts cause pulmonary fibrosis, silica is the dust par excellence predisposing to tuberculosis. This is doubtless due to its influence in forming a medium suitable not only for the survival but the proliferation of the tubercle bacillus in the lung (Kettle, private communication). The harmful

effects of soluble silica may be neutralised by simultaneous administration of basic dusts such as aluminium hydroxide or magnesium carbonate, though the latter are themselves harmful when inhaled alone. It is suggested that their respective solutes combine to form monosilicate. Monosilicates do not appear to have any harmful effect on the lung.

10. Heavy inhalations of any dust are liable to cause pulmonary damage.

11. The intensity of the initial pulmonary reaction to a dust is very generally in inverse ratio to the degree of eventual damage caused by the dust.

## REFERENCES.

- ARNOLD (1885). *Untersuchungen über Staubinhalation u. Staubmetastase*. Leipzig.  
 BADHAM (1927). *Rep. of Dir.-Gen. of Pub. Health for N.S.W.*  
 BEATTIE (1912). *Parl. Papers: Explosions in Mines. Committee Reports*, 1-6, 12.  
 BINET et CHAMPY (1926). *Comptes Rendus*, 94.  
 CARLETON (1924). *J. Hygiene*, 22.  
 — (1925). *Phil. Trans. Roy. Soc. B*, 83.  
 — (1927). *Quart. J. Micr. Sci.* 71.  
 — (1927). *J. Hygiene*, 26.  
 COLLIS and GILCHRIST (1928). *J. Ind. Hyg.* 10.  
 GRANEL et HEDON (1928). *Comptes Rendus*, 99, 22.  
 HAYNES (1926). *J. Hygiene*, 25.  
 HEFFERNAN (1929). *Brit. Med. Journ.* ii.  
 IRVING CLARK (1929). *J. Ind. Hyg.* 11, 3.  
 LANDIS (1925). *Ibid.* 7.  
 MAITLAND, CAVON and DETWEILER (1921). *J. Exp. Path.* 2.  
 MAVROGORDATO (1918). *J. Hygiene*, 17, 439.  
 — (1922). *Publicn. of S. Africa Inst. for Med. Res.* xv.  
 — (1926). *Ibid.* xix.  
 MIDDLETON (1929). *Brit. Med. Journ.* ii.  
 OPIE (1904). *Am. J. Med. Sci.* 127.  
 PERMAR (1920). *J. Med. Res.* 42.  
 POLICARD, DOUBROW et BOUCHARLAT (1929). *Bull. Hist. appl.* 6.  
 SEEMAN (1925). *Beitr. z. Path. Anat.* 74.  
 SEWELL (1918). *J. Path. and Bact.* 22, 40.  
 WAINWRIGHT and NICHOLS (1905). *Amer. J. Med. Sci.* 130, 403.  
 WESTHUES (1922). *Beitr. z. Path. Anat.* 70.  
 WILLIS (1922). *Amer. Rev. Tuberc.* 6.  
 WILLSON (1928). *Amer. J. Anat.* 41.

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# THE INCIDENCE OF CANCER OF THE BLADDER<sup>(1)</sup> AND PROSTATE IN CERTAIN OCCUPATIONS.

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## I. MATERIAL EXAMINED.

THE results given below are derived from an examination of death certificates of cases of cancer and papilloma of the bladder in both sexes, and of cancer of the prostate, from England and Wales between 1921 and 1928. The total numbers of certificates examined are shown in Table I; from the whole series of 13,965 cases those relating to cancer of the bladder in males, and cancer of the prostate,

Table I. *Death certificates examined.*

	Cancer of bladder	Papilloma of bladder	Cancer of prostate
<i>Males.</i>			
1921	589	86	—
1922	641	109	—
1923	647	114	—
1924	711	115	1022
1925	745	115	1116
1926	723	134	1145
1927	753	151	1172
1928	812	150	1353
	5621*	974	5808*
<i>Females.</i>			
1924	350	—	
1925	337	46	
1926	340	51	
1927	384	54	
	1411	151	

\* The difference between these totals and those given in Table II is due to the exclusion from the latter Table of seven cases of cancer of the bladder, and four cases of cancer of the prostate, occurring in persons under 20 years of age.

numbering 11,429, were chosen for study in detail, as in these classes the largest numbers and the best occupational data are available.

The two classes which were not used for exact tabulation of the occupations, namely, papilloma of the bladder, and cancer of the bladder in females, were put aside for the following reasons: (a) The cases of papilloma, which amount to little more than one-sixth of those of cancer of the bladder, are insufficient in number; but data were obtained from this series, in regard to the chemical trades especially, which will be studied in future when more figures are available. This class is valuable because the proportion of correct diagnoses is probably high and the inclusion of any prostatic or rectal tumours is unlikely. (b) The death certificates of women did not yield a sufficient number in which any industrial occupation was recorded. Thus, in 1925, only 15 of the 337 death certificates for cancer of the bladder in women recorded any occupation not connected with domestic, nursing or teaching work.

## II. SOURCES OF ERROR.

The analysis of such material as is afforded by the 11,429 certificates of cancer of the bladder in males, and of the prostate, encounters many difficulties, which may be classified under three headings.

First, it is necessary to know the total number of persons following any given occupation. This is learned from the Census results (in this case the Census of 1921) but (1) the figure given by the Census cannot be relied upon throughout the 10 years until the next Census; (2) the utility of the Census figures in the present investigation depends upon the possibility of obtaining in the death entries such detailed information in regard to occupation and industry as would enable the resultant figures to be related to the Census figures. It was not possible for example to ascertain a population at risk in regard to certain workers in chemicals and chemical manufactures in whom we were interested, and the corresponding death certificates, from which very interesting results might have been expected, could not be used statistically.

Second, with regard to the smaller occupations. Some of the most interesting from the present standpoint (patent fuel manufacture, tar distilling, chimney sweeping) employ a few thousand men only, who will yield but very few cases of cancer of the bladder even if the incidence is high. Hence the sampling error will be large even over a period of 8 years. Three of the four deaths of tar distillers from cancer of the bladder recorded in Table III occurred in two of the eight years (1922 and 1923).

Third, with regard to the individual entry in the death register. (1) This gives no information about the length of time during which the deceased followed the occupation named. (2) In some cases, especially in the case of deaths occurring in institutions, the information available to the Registrar at the time of registration is not so detailed as that supplied by the man himself at the Census. (3) Incorrect diagnosis is of course a possible source of error which is unavoidable in all such material.

In the work recorded here, an attempt has been made to avoid these inaccuracies by special enquiry into individual cases in some occupations (patent-fuel labourers, gas-works engine and crane drivers, gas fitters, gas stokers, gas-works inspectors and foremen, gas workers and labourers (not stokers), gas-producer men, coke-oven workers, brick kiln and oven men, blast furnacemen and labourers, dye mixers and dyers) where the smallness of the number employed made an error in a single death certificate most serious. Such enquiry was made in fifty cases of cancer of the bladder, chiefly of the more recent years (1924-1928), from relatives, fellow-workmen and employers of the deceased, with reference especially to the exact nature of the occupation and the length of time for which it had been pursued. Of these fifty cases, forty-four could be traced. In forty-one the reliability for our purpose of the information given on the death certificate was confirmed. In one case the short time for which the occupation had been followed (5 to 6 months) caused it to be rejected as a factor, although the entry was of course perfectly correct. In two cases the industry rather than the occupation in that industry had been recorded. Thus an "alkali labourer" was found to have worked for 32 years at a gas-producer plant, and the case was transferred accordingly to the class of gas-producer men.

The minimum duration of exposure necessary to produce an occupational cancer cannot be stated, and this question is a constant source of difficulty in such work as is presented here. In this series we have rejected the case of a gas-works labourer who had been employed in that work for not more than 5 or 6 months before death, and have retained the cases of a dyer with 2 years' and of a gas-producer man with 5 years' duration of employment before death.

The objection may be raised that if special enquiry be made into any of the cases in Table III it should be made into all. But (1) to investigate, in places scattered all over England and Wales, the accuracy of over 1400 death certificates was not practicable for us. (2) The risk of serious error is greatest in the smaller groups. Thus, if the certificate relating to one of four patent-fuel labourers is incorrect, the error is serious; but it is extremely unlikely that 25 per cent. of the 160 farmers are described incorrectly. (3) The comparatively small number of occupational terms in some of the larger groups causes less classification difficulty in those groups than in some of the smaller ones. Thus, an agricultural labourer can hardly be misnamed, whereas a tar distiller may be described as a by-product worker, or a stillman in a chemical works.

The occupational classification in use at the General Register Office was revised in 1921. The change was made in accordance with a resolution of the British Empire Statistical Conference of 1920 in favour of separate and independent tabulations by occupation and by industry. The classifications used in 1911 and earlier were only in part occupational, being largely industrial in nature. The change precludes the possibility of an exact comparison with previous Census results (see *General Report, Census of England and Wales, 1921*, p. 86), or with the data on the occupational incidence of cancer in England and



Wales in 1910, 1911 and 1912, published by the Medical Research Council (Young, Russell, Brownlee and Collis, 1926). In the last-named report the occupations associated with coal gas, tar and pitch, do not appear in the tables devoted to cancer of the bladder and prostate.

### III. METHOD OF CALCULATION.

In estimating the occupational incidence of any form of cancer it is, of course, necessary to correct for age distribution, for an occupation employing a large proportion of older men will yield more cases of cancer apart from any aetiological factor. The Census Occupation Tables give the age distribution, in decennial periods, of the whole population of males, and also of those following each one of the recognised occupations, at the time of the Census. The number of cases of cancer of the bladder and of the prostate occurring in the whole male population in each of these decennial periods during the years (Table I) in question was obtained from material at the General Register Office. The comparison of the various occupations with the general population is then a sum in proportion. In Table II the method of calculation is shown in detail by three

Table II. *Cancer of bladder and prostate.*

	Age group							Total aged 20 and upwards	Total registered deaths	Ratio of registered to 100 calculated deaths
	20-	25-	35-	45-	55-	65-	70 and upwards			
All males										
Population	1,448,385	2,621,280	2,496,375	2,133,179	1,382,843	449,363	530,867	11,062,292	—	—
Deaths: Bladder	10	28	176	668	1621	1116	1995	5614	5614	100
Prostate	4	3	28	256	1338	1255	2920	5804	5804	100
Farmers										
Population	7,358	37,688	57,806	63,325	52,317	19,694	29,405	267,593	—	—
Deaths: Bladder	0.0	0.4	4.1	19.8	61.5	48.9	110.5	245.2	160	65
Prostate	0.0	0.0	0.6	7.6	50.6	55.0	161.8	275.6	236	86
Tar-Distillery Workers										
Population	266	595	622	594	399	123	66	2,665	—	—
Deaths: Bladder	0.0	0.0	0.0	0.2	0.5	0.3	0.2	1.2	4	333
Prostate	0.0	0.0	0.0	0.1	0.4	0.3	0.4	1.2	1	83
Patent-Fuel Labourers										
Population	313	532	444	306	143	44	29	1,811	—	—
Deaths: Bladder	0.0	0.0	0.0	0.1	0.2	0.1	0.1	0.5	2	400
Prostate	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.4	1	250

examples, namely, one large class (farmers), and two very small ones (tar-distillery workers, labourers at patent fuel works). All the ratios given in Table III were obtained in this way.

Table II shows, for example, that all the 2,133,179 males in England and Wales between the ages of 45 and 55 produced 668 fatal cases of cancer of the bladder in the years 1921 to 1928. The 63,325 farmers in this age-group should, at this rate, give 19.8 cases. One proceeds in this way through each age-group and adds up the total cancers of the bladder for a hypothetical general population of the number and age distribution of farmers, obtaining a total of 245.2. But the actual number given by the death certificates of farmers is 160. The ratio for farmers is therefore  $\frac{160 \times 100}{245.2} = 65$ .

## IV. INCIDENCE OF CANCER OF THE BLADDER.

The occupations named in Table III employed, at the time of the Census in 1921, 3,042,990 out of the 11,062,292 males aged 20 and upwards, and they include most of the men engaged in the two largest industries in the country, namely agriculture and coal-mining. The Table includes 1456 out of the 5614 cases of cancer of the bladder, and 1593 out of the 5804 cases of cancer of the prostate, occurring in the whole population in question. These figures for the three totals, and the fractions of these three totals which are dealt with in Table III show nearly the same ratio; thus

$$\frac{11,062,292}{3,042,990} = 3.6, \text{ and } \frac{5614}{1456} = 3.8, \text{ and } \frac{5804}{1593} = 3.6.$$

Of course, many other occupations might have been considered, but the object of this investigation was primarily the incidence of cancer of the bladder upon workers in the gas, tar and chemical industries.

The results given in the table suggest the following comments:

(1) The ratio in all the open-air occupations not exposed to industrial vapours and dusts (gardeners, farm bailiffs and foremen, agricultural labourers and shepherds, farmers) which together include over 920,000 men, is low (87, 74, 65, 65).

(2) The horse drivers, grooms and horse keepers show likewise low ratios (88, 62), though many of these men must be town-dwellers.

(3) The coal-mining industry shows a ratio below 100 in five out of six subdivisions (workers above ground 85, hewers 81, persons making and repairing roads 80, other workers below ground 53). The exceptional group is that of "persons conveying material to the shaft," with a ratio of 171. Probably this high figure is due to lack of conformity between the descriptions of occupation given by the men themselves in the Census schedules and the description obtained by the Registrar at the time of death, and is not to be taken as reliable. The idea suggests itself that these persons engaged in the traction work of the mine are most exposed to lubricating oil, but this matter would need special enquiry. If the three groups of underground workers other than hewers (persons conveying material to the shaft, persons making and repairing roads, other workers below ground) are pooled, a total of fifty-four deaths is obtained with a ratio of 78.

(4) The table shows that, out of the forty-six occupations examined, fifteen have ratios for cancer of the bladder greater than 150. These fifteen occupations may now be considered in some detail. In five, and possibly in eight, of them there is exposure to coal gas, tar, or pitch; in one to lubricating oil; and in two to tobacco.

(a) Five of the fifteen occupations (gas stokers, gas fitters, tar distillers, gas-works engine and crane drivers, patent-fuel labourers) involve exposure to coal gas, tar, or pitch, the last three of these occupations showing the highest ratios on the list, namely 333, 400 and 400. The numbers of deaths in some of

Table III. *Cancer of bladder and prostate.*

The occupations are placed in the descending order of the ratio of registered to 100 calculated deaths from cancer of the bladder.

	Population Age 20 and upwards	Calculated deaths		Registered deaths		Ratio of registered to 100 calculated deaths	
		Bladder	Prostate	Bladder 1921-28	Prostate 1924-28	Bladder	Prostate
Labourers at patent fuel works ...	1,811	0.5	0.4	2	1	400	250
Gas-works engine and crane drivers...	1,915	1.0	—	4	None	400	—
Tar-distillery workers... ..	2,665	1.2	1.2	4	1	333	83
Cellarmen ... ..	5,844	2.6	2.5	6	5	231	200
Lithographic and process engravers...	4,440	1.3	1.2	3	2	231	167
Tobacco manufacture... ..	7,309	2.7	2.7	6	5	222	185
Gas-works managers ... ..	1,614	0.9	0.9	2	3	222	333
Gas fitters ... ..	11,343	3.9	3.8	8	8	205	210
Brick kiln and oven men ... ..	5,143	2.0	1.8	4	4	200	222
Cotton spinners and piecers ...	32,448	10.6	9.7	21	14	198	144
Coal miners—persons conveying ma- terial to the shaft ... ..	72,821	8.2	6.2	14	6	171	97
Tanners, leather dressers, curriers; skilled workers ... ..	18,372	10.1	10.4	17	15	168	144
Brick and plain tile makers ... ..	7,868	3.8	4.1	6	4	158	98
Gas stokers ... ..	11,931	4.5	3.5	7	8	156	229
Tobacconists and their assistants ...	14,470	7.2	7.3	11	9	153	123
Painters ... ..	159,809	78.2	74.8	115	105	147	140
Dye mixers and dyers ... ..	19,423	8.3	7.8	12	12	145	154
Blast furnace enginemmen ... ..	1,531	0.7	—	1	None	143	—
Gas-producer men ... ..	4,112	2.1	1.7	3	2	143	118
French polishers ... ..	15,048	5.6	5.2	7	10	125	192
Licensed victuallers ... ..	75,038	54.3	54.1	67	75	123	139
Coke-oven workers ... ..	9,318	4.9	3.8	6	1	122	26
Hairdressers ... ..	30,943	10.2	9.0	12	9	118	100
Bakers and pastrycooks ... ..	56,603	26.2	26.3	31	24	118	91
Gas workers and labourers (not stokers) ... ..	32,761	17.9	17.4	21	22	117	126
Stationary engine and crane drivers	79,226	35.8	33.9	40	35	112	103
Carpenters ... ..	188,199	123.6	133.0	134	114	108	86
Cabinet makers ... ..	31,770	18.3	19.7	19	16	104	81
Printers ... ..	75,454	31.1	29.5	32	32	103	108
Chimney sweeps ... ..	5,274	4.5	4.9	4	3	89	61
Drivers of horse-drawn vehicles ...	178,421	81.8	78.6	72	62	88	79
Gardeners ... ..	181,112	164.0	184.2	143	204	87	111
Coal miners—workers above ground	83,338	42.5	41.8	36	46	85	110
Gas-works inspectors and foremen ...	4,668	2.4	2.2	2	1	83	45
Coal miners—hevers ... ..	487,304	139.3	126.7	125	114	81	90
Coal miners—persons making and re- pairing roads ... ..	54,696	27.5	26.2	22	20	80	76
Cotton weavers ... ..	38,928	16.7	16.6	13	14	78	84
Farm bailiffs and foremen ... ..	23,129	13.5	13.6	10	21	74	154
Plumbers ... ..	41,336	15.5	14.4	11	8	71	56
Agricultural labourers, including shep- herds... ..	449,587	294.4	319.7	191	231	65	72
Farmers ... ..	267,593	245.2	275.6	160	236	65	86
Grooms and horsekeepers ... ..	36,055	21.0	21.2	13	22	62	104
Locomotive engine drivers, firemen and cleaners... ..	88,009	28.6	27.0	16	33	56	122
Coal miners—other workers below ground ... ..	79,174	33.9	30.9	18	30	53	97
Blast furnacemen and labourers ...	13,363	6.0	5.7	3	4	50	70
Barmen ... ..	16,112	4.1	3.3	2	2	49	61
Total... ..	3,042,990			1456	1593		

these occupations is very small, and is therefore liable to a large sampling error, but the concordant tendency of the evidence from the five groups seems to have some cumulative weight. The only branches of the gas industry which have lower ratios are (1) "Gas workers and labourers (not stokers)" (ratio 117), who include probably a number of unskilled labourers who may not have been long in this employment; and (2) "Gas-works inspectors and foremen" (ratio 83), who are represented by two persons only, a gas-works foreman and a gas-meter inspector. The occupation of gas-works engine and crane driver is included here because these men would be exposed to the volatile products of the gas works.

The sixth group from the coal gas industry showing a ratio above 150 is that of gas-works manager (ratio 222), but we do not know whether the two persons represented in the table had been subjected to the atmosphere of the gas works. This group gives also the highest figure in the table (ratio 333) for cancer of the prostate.

Of the four brick kiln and oven men (Table III, p. 130) one was described at death as a kiln setter, and investigation showed that setting kilns had been his main occupation. The second was described as a kiln man; on investigation he was found to have been so employed for the last 5 years; previously he had been a brick setter for 10 years, and before that on various jobs in the works for 15 years. The third and fourth were described as brick burners, but one of these had been mainly a kiln setter, flue cleaner, and in his spare time a chimney sweep, while the other was known to have been at the brick works all his working life, of which the last 10 years were certainly spent in brick burning only.

The presser comes into contact with "brick oil" or "moulding oil," which consists mainly of the creosote fraction of coal tar. The setter, in handling the bricks after moulding, is also exposed to the same oil, but in a warmer atmosphere when inside the kiln. Hence in three, if not four, of these cases prolonged contact with creosote oil is at least probable.

During the period in question (1921-8) there was also one death from cancer of the bladder in a stationary engine man at a creosote works. The age distribution of the men in this occupation is not available; hence the case does not appear in Table III.

(b) One occupation is associated certainly with exposure to lubricating oil, namely that of cotton spinners and piecers (ratio 198). The great liability of cotton spinners to occupational epithelioma of the skin due to mineral oil is of course well known. Table IV shows that in six years no less than 463 cases of cancer of the skin were notified in cotton mule spinners, while in the same time five cases only were notified in cotton weavers. With these numbers one may compare those in Table III for cancer of the bladder which show a high ratio (198) for cotton spinners and piecers, and a low one for cotton weavers (78). The numbers engaged in the two occupations are not very different (spinners and piecers 32,448; weavers 38,928). Possibly the incidence of cancer of the bladder upon cotton spinners and piecers is even understated here,

Table IV. *Showing the industries in which occurred the 546 cases of skin epithelioma notified as due to shale oil or mineral oil from 1923 to 1928 inclusive (England and Wales).*

Industry	Total	Industry	Total
Shale oil refining ... ..	30 (1920 to 1928)	Woollen ... ..	3
Cotton: Blowing ... ..	1	Jute ... ..	1
Carding ... ..	7	Engineering ... ..	20
Mule spinning ... ..	463	Coal mining, pump oiler...	1
Ring spinning ... ..	2	Oil blending ... ..	3
Doubling ... ..	1	Matches, oil storeman ...	1
Weaving ... ..	5	Gunsmith ... ..	1
Winding ... ..	1	Brewing engineer... ..	1
Oiler and greaser... ..	1	Stone sawing, crane driver	1
Engine tender ... ..	1		
Engineer ... ..	2		
		Total... ..	546

Table V. *Showing the industries in which occurred the 439 cases of skin epithelioma notified as due to pitch, tar or tarry products from 1920 to 1928 inclusive (England and Wales).*

Industry	Total	Industry	Total
Patent fuel manufacture ...	183	Cable manufacture ... ..	6
Tar distilling ... ..	118	Cresosoting timber ... ..	5
Coal gas manufacture ... ..	65	Anthracene manufacture ...	4
Pitch loading (wharfs, etc.)	22	Net fixing ... ..	3
Coke ovens ... ..	8	Barge repairing ... ..	2
Brick, tile and pipe manufacture	6	Felt proofing ... ..	2
Producer gas manufacture ...	6	*Other industries ... ..	9
		Total... ..	439

\* Include manufacture of brattice cloth 1, asphalt troughs 1, electric brushes 1, bitumastic paint 1, sails 1, insulators 1, and the occupations of creosoting moulds (steel works) 1, cable handling (telegraph construction) 1, and roadman (local councils) 1.

because during the last few years the cotton trade has been depressed and men in the earlier stages of the disease may have taken to other occupations, which would be entered upon the death certificates.

(c) Both occupations connected with tobacco give rather high ratios (tobacco manufacture 222, tobaccoists and their assistants 153).

(d) Cellarmen show a high ratio (231), but another occupation associated with alcohol (barmen) has a very low one (49), and that of licensed victuallers a ratio near the normal level (123).

(e) The two remaining classes with ratios above 150 (lithographic and process engravers 231, tanners, leather dressers and curriers 168) are both concerned with work which is chemical in character.

(5) Gas-producer men show a ratio of 143. Neither coke-oven workers (122) nor chimney sweeps (89) have a high ratio, though both are liable to cancer of the scrotum. The very widespread use of producers is a recent development and there must be many workers who have not been very long in this employment. Exposure to lubricating oil does not always give a high ratio, for locomotive drivers, firemen and cleaners show a low one; but probably this class has a high standard of personal cleanliness. Also, locomotive drivers are under constant medical inspection and are drafted to other work if they show any failure of stamina, and hence the deaths of such men will be assigned to other occupa-

tions. Blast furnacemen and labourers show a low ratio (50); two of the three cases were traced by enquiry, and the occupation of one of these was found to have been that of a labourer and gas stoker at a gas works maintained for producing ordinary coal gas to light the blast furnace.

This section may be summarised by bringing together from Table III the figures relating to the eleven occupations which may involve exposure to coal gas, tar, pitch or soot. The ratio in nine of these is above 100, and in six is above 150.

*Cancer of bladder.*

	No. of deaths	Ratio
Patent-fuel labourers ... ..	2	400
Gas-works engine and crane drivers ...	4	400
Tar-distillery workers ... ..	4	333
Gas-works managers ... ..	2	222
Gas fitters ... ..	8	205
Gas stokers ... ..	7	156
Gas-producer men ... ..	3	143
Coke-oven workers ... ..	6	122
Gas workers and labourers (not stokers)	21	117
Chimney sweeps ... ..	4	89
Gas-works inspectors and foremen ...	2	83

V. OCCURRENCE OF TUMOURS OF THE SKIN, AND OF THE BLADDER,  
IN THE SAME PERSON.

Workers exposed to coal tar and pitch may develop papilloma or carcinoma of the skin before a tumour of the bladder is known to be present. By special enquiry into case histories we have found two instances of this in our own series (Table III, p. 130), and we have learned of two more from the literature. Among our own cases: (1) In a patent-fuel worker papillomata of the face had been diagnosed four times between 1916 and 1926; in 1927 he died of cancer of the bladder. (2) The other patent-fuel worker suffered from an "ulcerating wart" on the wrist in 1921 and died of cancer of the bladder in 1928. (3) In a German journal (Posner, 1898) there is an extremely scanty report of the co-existence of a bladder tumour with a scrotal cancer in a tar worker ("Herr Posner (Berlin) hat zweimal Blasentumoren bei Arbeitern aus einer Naphthol resp. Theerfabrik beobachtet, im letzten Fall gleichzeitig mit einem typischen Skrotaalkrebs"). (4) O'Donovan (1920) records a case admitted to the London Hospital. "S. V., aged 67 years, worked in tar for 26 years, and after 11 years developed 'eczema' and warts on the arms and legs. Four and two and a half years before his admission he was operated on for 'tar warts,' and seven weeks before his admission he had haematuria, which on cystoscopy... proved to be due to an inoperable carcinoma of the bladder." Elsewhere in the paper this man is described more exactly as a tar distiller.

These cases are of interest also in connection with the possibility that a protective action against the development of cancer in any one part of the body may be exerted by an existing benign or malignant tumour in another part (cf. Murray, 1923). The prospect here does not seem very hopeful.

VI. THE RELATIONS BETWEEN CANCERS OF THE SCROTUM,  
OF OTHER PARTS OF THE SKIN, AND OF THE BLADDER.

It is perhaps permissible to attempt, in a purely tentative way, some correlation between the data given in Table III and those which we possessed previously about occupational cancers. There appear to be some curious differences in the incidence of these cancers; Table VI is intended to present, not any conclusive results, but suggestions for the further investigation of the apparent differences. These may be real, or may be due to inadequacy of the data, especially in regard to cancer of the bladder; in either case they show the need for much further research upon industrial cancer.

Table VI.

	Cancer of					
	Scrotum		Other skin			Bladder
Pitch workers	+	+	+	+	+	+
Tar workers	+		+	+		+
Chimney sweeps	+	+		+		-
Mule spinners	+	+		+		+
Shale-oil workers	+		+	+		-
Chemical workers	-			-		+
Gas-producer men	+			-		-
Coke-oven men	+	+		+		-

The signs + and - are used here not in any exact qualitative sense, but to give a rough quantitative expression of such abundance or rarity as is suggested by the available data derived from deaths or notifications during life. The proportions indicated by single or multiple + signs in the first two columns of the table are based on data given in earlier papers (Kennaway, 1925; Bridge and Henry, 1928; Henry, 1928), which need not be set out again here, and on material as yet unpublished. Thus, in mule spinners about two-thirds of cancers of the skin occur on the scrotum and one-third elsewhere, while in the tar workers this relation is reversed. Dr Alexander Scott, whose monograph (1923) contains practically everything that is known about cancer in the shale oil industry, has been so kind as to inform us that in his 35 years' experience he has never known a case of cancer of the bladder in a shale-oil worker, but no special enquiry, such as the systematic searching of local death registers, has been made on this point. By "chemical workers" here is meant a large class including those generally spoken of by writers on cancer, often on very inadequate grounds, as "aniline dye workers."

The apparent absence of occupational cancer of the bladder in the smaller groups (shale-oil workers, sweeps) may be due to the much less frequent occurrence of this form of cancer, in comparison with that of the skin. This is shown by the figures given in Table VII which are derived from the series in Tables III, IV and V.

Table VII.

		Notified cases of cancer of skin	Fatal cases of cancer of bladder
1921-1928	Patent fuel manufacture	153	2
	Tar distilling	114	4
	Mule spinning	463 (1923-1928)	21 (1921-1928)

The results suggest the idea that the skin serves as a very effectual first line of defence to the urinary tract; but of course the substances producing cancer of the skin, and of the bladder, may be different, and the latter may enter through the respiratory or alimentary tract. The whole group of phenomena summarised, very inadequately, in Table VI requires much further investigation.

## VII. INCIDENCE OF CANCER OF THE PROSTATE.

The examination of the death certificates of cases of cancer of the prostate was intended to provide a control upon those of cancer of the bladder, in which an occupational factor was probable; thus it was hoped that the occupations showing an exceptionally high incidence of bladder cancer would show a more or less normal incidence of prostatic cancer. But the selection of cancer of the prostate as a control was probably an unfortunate one, for two reasons. First, the prostate may not be altogether immune from the liability to industrial cancer which affects the upper parts of the urinary tract. This possibility has been suggested by Oppenheimer (1926), who has recorded a case of cancer of the prostate, and one of cancer of the posterior part of the urethra, in chemical workers. Second, cancer of the prostate may be mistaken for cancer of the bladder, or *vice versa*, more especially where the medical attendant has no opportunity for special knowledge of urology, nor for the performance of autopsies; under these conditions the more likely of the two possible errors is that cancer of the prostate will be recorded as cancer of the bladder.

It is difficult to decide which organ, other than the prostate, would have been most suitable as a control unaffected by occupational conditions. The whole respiratory tract, and the upper alimentary tract, including the stomach, are obviously unfitted for this purpose, while the recorded number of cases of intestinal cancer may be affected considerably by errors of diagnosis. Probably cancer of the rectum would have provided the best control figures, for in this case the influence of external factors is improbable, and the diagnosis will be generally correct.

Some comments may be made upon the figures for cancer of the prostate given in Table III (p. 130).

(1) The three occupations which show the highest ratios for cancer of the bladder (patent-fuel labourers, 400; gas-works engine and crane drivers, 400; tar-distillery workers, 333) show for cancer of the prostate either no deaths at all, or a low ratio (tar-distillery workers, 83) or a fairly high one (patent-fuel labourers, 250). Hence, if any weight at all is to be attached to these figures, they indicate that these occupations predispose to cancer of the bladder rather than to cancer of the prostate. One notes also that the coke-oven workers produced six cancers of the bladder and one cancer of the prostate, although the latter disease is about 1.6 times as common as the former in the general population (Table I).

(2) But the matter is not very simple, for in the succeeding part of Table III



are several occupations in which the ratio is high for both forms of cancer. Thus:

			Ratio	
			Bladder	Prostate
Cellarmen	...	...	231	200
Gas-works managers	...	...	222	333
Gas fitters	...	...	205	210
Brick kiln and oven men	...	...	200	222
Gas stokers	...	...	156	229

And it is noteworthy that the cotton spinners show again a higher ratio than do the cotton weavers, although the difference is not so great as in the case of the bladder.

There seems to be nothing to help one in deciding between the various possible factors (occupational incidence upon both organs, errors of diagnosis, errors of sampling) which might be suggested to explain the above figures.

(3) The remaining occupations, with ratios for cancer of the bladder below 150, show on the whole fairly close agreement between the two series (bladder and prostate). Thus the ratios for bladder and for prostate differ by not more than twenty in the following fifteen classes: painters, gas-producer men, licensed victuallers, hairdressers, gas workers and labourers (not stokers), stationary engine and crane drivers, printers, drivers of horse-drawn vehicles, coal miners (hewers, and persons making and repairing roads), cotton weavers, plumbers, agricultural labourers and shepherds, blast furnacemen and labourers, barmen.

It is noteworthy that, in the open-air group (gardeners, farm bailiffs and foremen, agricultural labourers and shepherds, farmers), the ratio for bladder is throughout lower than that for prostate. In the wood-working group (carpenters, cabinet-makers) the reverse is seen. High figures for prostate, in comparison with those for bladder, are given by French polishers, farm bailiffs and foremen, and locomotive engine drivers, firemen and cleaners.

#### VIII. CANCER OF THE BLADDER AND PROSTATE IN CHEMICAL WORKERS.

Our series includes 61 fatal cases of cancer of the bladder in chemical workers (dyers, dye mixers, workers in rubber and explosives, and those engaged in all unspecified chemical processes). In only one of these groups however, namely that of the dyers and dye mixers, was it possible to ascertain the population at risk, and hence these alone of the chemical workers appear in Table III. But we have accumulated, by special enquiry into individual cases, a large amount of data upon the actual processes in which the other chemical workers were engaged, and we are utilising this information as a guide in experimental work, and in other ways.

#### IX. SUMMARY.

In eight out of ten occupations associated with exposure to coal gas, tar, pitch or soot the incidence of cancer of the bladder is greater than that found

in the general male population, and in five of the ten it is from one and a half to four times as great. Three of these occupations show the highest figures for incidence of cancer of the bladder observed among the forty-six occupations investigated. Various possible sources of error in these figures are discussed, of which the most serious is the smallness of the numbers of deaths involved. The corresponding data for cancer of the prostate give less consistent indications of an occupational liability. The subject needs much further enquiry, but it has seemed best to publish now, without further delay, such material as is available, rather than wait for another long term of years while more data accumulate.

A large, and quite essential, part of this investigation, namely the computing of the population for each occupation recorded on the death certificates, and the calculation of the ratios, has been carried out by members of the staff of the Registrar General, to whom we wish to express our great indebtedness. We have to thank the British Empire Cancer Campaign for a grant which enabled the special enquiry into individual cases to be made, and to record our gratitude to those who assisted so willingly in carrying out this enquiry.

#### REFERENCES.

- BRIDGE and HENRY (1928). *Report Internat. Conference on Cancer, London*, p. 258.  
 HENRY (1928). *J. Hygiene*, **28**, 100.  
 KENNAWAY (1925). *J. Industr. Hygiene*, **7**, 69.  
 MURRAY (1923). *8th Scientific Report Imper. Cancer Res. Fund*, p. 75.  
 O'DONOVAN (1920). *Brit. J. Dermatol. and Syph.* **32**, 215.  
 OPPENHEIMER (1926). *Deutsche med. Wochenschr.* **52**, 1342.  
 POSNER (1898). *Ibid.* (Vereins Boilage), **24**, 210.  
*Report of the Departmental Committee appointed to consider evidence as to the Occurrence of Epitheliomatous Ulceration among Mule Spinners.* H.M. Stationery Office, London, 1926.  
 SCOTT (1923). *8th Scientific Report Imper. Cancer Res. Fund*, p. 85.  
 YOUNG, RUSSELL, BROWNLEE and COLLIS (1926). *An Investigation into the Statistics of Cancer in different Trades and Professions.* London: H.M. Stationery Office.

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# FURTHER EXPERIENCE OF THE BISMUTH SULPHITE MEDIA IN THE ISOLATION OF *BACILLUS TYPHOSUS* AND *B. PARATYPHOSUS* B FROM FAECES, SEWAGE AND WATER.

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## INTRODUCTION.

In two previous papers (1927 and 1928) we have described and applied a new medium for the isolation of *B. typhosus* from faeces and sewage. Favourable results on the use of the medium for the isolation of the *B. typhosus* from the stools of patients and "carriers" have been reported by Allison (1927-8), Mazzetti (1928) and D'Antona (1929), for the isolation of *B. typhosus* from the droppings of seagulls by Adams (1928), for the isolation of *B. typhosus* from sewage and cockles by Wilson (1928), for the isolation of *B. typhosus* from

water by A. C. Houston (1928 and 1929) and Franco (1929), and for the isolation of *B. paratyphosus* B from Edinburgh sewage by Gray (1929) and by Begbie and Gibson (1930). The present paper is divided into three parts. Part I deals with further experience with the same media as described by us in 1927. In Part II modifications of these media are described and tested; and in Part III a description is given of a fluid bismuth sulphite enrichment medium which promises to be even more efficient than our solid media for the isolation of typhoid bacilli from the stools of convalescent typhoid fever cases.

### PART I.

No modification of the media has been found necessary since our original publication (1927), but we may supplement our description of the media with the following remarks: (1) No filtration of the 3 per cent. agar is required; the medium is made in 12-litre amounts in a preserving pan in an autoclave and, after cooling and solidifying, the pan being inverted, the agar is turned out, and the lower layer containing precipitated matter is cut away from the clear upper layer. (2) The 1 gram. of exsiccated sodium phosphate which is added to each 100 c.c. of the medium is dissolved in 10 c.c. of boiling water. (3) Instead of a 20 per cent. solution of exsiccated sodium sulphite a 40 per cent. solution of sodium sulphite crystals may be used.

The original description of the media was:

Medium A. To 100 c.c. of a melted 3 per cent. nutrient agar are added 5 c.c. of a 20 per cent. solution of glucose, 10 c.c. of a 20 per cent. solution of sodium sulphite (anhydrous), 5 c.c. of a standard bismuth solution. After boiling for 2 minutes an addition is made of 1 gram. of exsiccated sodium phosphate and 1 c.c. of an 8 per cent. solution of ferrous sulphate crystals.

Medium B. The same as medium A with the addition of 0.5 c.c. of a 1 per cent. watery solution of brilliant green. The standard liquor bismuthi is prepared by mixing 60 gram. bismuth citrate with 50 c.c. of distilled water, and then with 20 c.c. liq. ammonii sp. gr. 0.880, and finally making the volume up to 500 c.c. with distilled water.

The following details in making the liquor should be followed as they ensure the action to the full of the ammonia employed. A glass-stoppered bottle is almost filled with 500 c.c. of distilled water, and the level of the fluid is marked on the side of the vessel. The water is poured out, 60 gram. of bismuth citrate are introduced through a wide funnel and followed by 50 c.c. of distilled water. By means of a glass rod the citrate is made into a thin paste with the water, and then 20 c.c. of liq. ammonii are poured in. The mixture is stirred with the glass rod and a chemical reaction occurs with the development of heat. The glass stopper is inserted, the bottle shaken, and as soon as the bismuth citrate is almost completely dissolved, distilled water is added until the level reaches the 500 c.c. mark.

The principle of the medium rests (1) on the positive property of the *B. typhosus* being able to reduce a sulphite to a sulphide in the presence of

glucose; (2) on the inhibitory action on the growth of *B. coli* and of many other bacteria, of a bismuth sulphite in the presence of a certain excess of sodium sulphite.

ISOLATION OF *B. TYPHOSUS* FROM FAECES.

Since the publication of our last paper we have had an opportunity of examining the stools of 50 different cases of typhoid fever, and in 44 we have

Table I.

No.	Date	Patient's identification sign	No. of typhoid colonies found on			
			MacCon- key medium	MacCon- key plus brilliant green	Bismuth medium (A)	Bismuth plus brilliant green (B)
1	28. v. 27	McQ.	0	—	6	10
2	28. v. 27	B.	0	—	24	24
3	28. v. 27	F.	5	—	2000	1500
4	31. v. 27	L. J.	0	—	200	400
5	31. v. 27	M. J. P.	5	—	100	350
6	9. vii. 27	McB.	0	400	1000	1000
7	9. vii. 27	I.	3	5	148	132
8	13. vii. 27	M. D.	5	—	600	600
9	25. vii. 27	S. P.	0	3	6	6
10	28. vii. 27	R. S.	0	—	5	9
11	29. viii. 27	M. McR.	—	—	2	3
12	7. ix. 27	G.	6	—	6000	6000
13	16. ix. 27	R.	0	—	2	2
14	19. ix. 27	M. D.	0	200	200	200
15	20. x. 27	M.	2	6	300	200
16	8. xi. 27	F. R.	12	180	180	110
17	28. xii. 27	W. A.	0	—	500	400
18	18. i. 28	K. E.	30	90	600	500
19	21. ii. 28	A. S.	0	—	2	2
20	21. ii. 28	T. McM.	8	—	600	600
21	18. ix. 28	J. M.	0	2	0	20
22	22. ix. 28	E. McM.	0	20	300	300
23	10. x. 28	McB.	80	100	600	400
24	16. iii. 29	M. B.	0	—	0	Hs.*
25	21. iii. 29	B. T.	0	—	Hs.	Hs.
26	4. v. 29	M. E.	0	—	57	120
27	20. vi. 29	E. McG.	0	—	9	5
28	2. vii. 29	A. T.	0	—	0	5
29	7. ix. 29	J. O'H.	0	—	20	1000
30	13. ix. 29	M. D.	0	1	0	500
31	30. i. 30	N. D.	500	500	500	500
32	17. ii. 30	D. H.	0	0	70	60
33	20. iii. 30	J. A.	20	60	60	33
34	29. iii. 30	M. B.	0	0	16	24
35	1. iv. 30	J. Ma.	1	0	200	200
36	9. iv. 30	J. M.	36	40	500	500
37	12. iv. 30	H.	0	0	0	0
38	12. iv. 30	M. By.	2	0	20	3
39	12. iv. 30	C. M.	0	0	0	0
40	24. v. 30	P. R.	200	1000	1000	1000
41	27. v. 30	N. P.	1	500	500	500
42	9. v. 30	N. Bi.†	0	0	0	0
43	9. v. 30	Mrs Bi.†	0	0	0	0
44	14. vi. 30	A. McL.	0	0	0	0
45	14. vi. 30	F. McL.	0	0	0	0
46	13. vii. 30	J. R.	0	Hs.	Hs.	Hs.
47	1. vii. 30	M. R.	0	Hs.	Hs.	Hs.
48	1. vii. 30	M. S.	0	Hs.	0	Hs.
49	26. vii. 30	M. E.	0	24	100	300
50	19. viii. 30	E. I.	0	—	—	30

\* Hs. = hundreds.

† Convalescent.

been successful in isolating the *B. typhosus* by means of the bismuth media. In most of the tests we also employed MacConkey's medium and MacConkey's medium containing brilliant green. Table I shows the approximate numbers of typhoid bacilli which developed on plates of the different media when inoculated with a drop of an emulsion of the typhoid stool.

With the MacConkey bile salt lactose neutral red agar medium on 17 occasions out of 49 attempts, *B. typhosus* was isolated, *i.e.* 34.7 per cent.; with MacConkey media containing in each 100 c.c. 0.3 c.c. of a 1 per cent. solution of brilliant green, 20 out of 30 attempts were successful, *i.e.* 66.6 per cent.; similarly with bismuth medium A, 38 out of 49, *i.e.* 77.5 per cent., and with bismuth medium B, 44 out of 50 attempts, *i.e.* 88 per cent. were successful.

Table I also shows that, as a rule, the typhoid colonies developing on the bismuth plates were several hundred times more numerous than those developing on the MacConkey medium. This is undoubtedly due to the suppression of the *B. coli*. Where the growth of this bacillus is suppressed by brilliant green, then the number of colonies on the MacConkey brilliant green plates and on the bismuth plates is much the same. With media depending for the differentiation between colonies of *B. typhosus* and *B. coli* on the presence of lactose, the dice are loaded against *B. typhosus*, since an extra supply of energy in a utilisable form is being supplied to the *B. coli*. It is also known (Nissle, 1916; Smith, 1923; Hashimoto, 1927; Vignati, 1928) that certain strains of *B. coli* are actively antagonistic to the growth of *B. typhosus*, as well as being able by the rapidity of their growth to occupy by their colonies nearly all available territory on a plate.

#### ISOLATION OF *B. PARATYPHOSUS* B FROM FAECES.

In Table II are tabulated the results of the examination of stools from 36 cases of paratyphoid fever. In all these the infective agent was the *B. paratyphosus* B.

The growth of *B. paratyphosus* B is for about 48 hours suppressed on the bismuth media, and as at the end of that time colonies of *B. coli* will in many cases have developed on bismuth medium A, it is better to use bismuth medium B containing brilliant green.

After 48 hours the colonies of *B. paratyphosus* B may appear black and not unlike those of *B. typhosus*, but slightly more raised and a little moister; more frequently they appear as moist, raised, clear, sticky colonies which, after another 24 hours, become black and also blacken the surrounding medium. On further incubation the colonies continue to enlarge until they may be 0.6 to 1 cm. in diameter. At this stage the colony is dark green in colour, the margin is raised, and the appearance resembles that of a motor-wheel disc surrounded by its tyre.

For prompt isolation of the *B. paratyphosus* B the bismuth media are not suitable, but, from our experience, we consider that medium B should always be employed in the examination of enteric stools as, occasionally,

*B. paratyphosus B* will be obtained with it where failure has occurred with the brilliant green MacConkey medium, by means of which, as a rule, the isolation is quick and satisfactory. With the MacConkey medium 11 out of 19 tests were positive, i.e. 58 per cent.; with MacConkey brilliant green medium 22 out of 26 tests were positive, i.e. 84.6 per cent. With the bismuth medium A, 19 out of 22 tests were positive, i.e. 86.3 per cent. and, with bismuth medium B, 22 out of 27 were positive, i.e. 81.5 per cent.

Table II.

No.	Date	Patient's identification sign	No. of paratyphoid colonies found on			
			MacCon- key medium	MacCon- key plus brilliant green	Bismuth medium (A)	Bismuth plus brilliant green (B)
1	7. vii. 27	P. N. T.	Hs.	—	Hs.	Hs.
2	20. vii. 27	D. McC.	0	—	1	33
3	20. vii. 27	E. Y.	5	—	5	300
4	23. iii. 28	D. B.	0	17	21	—
5	1. iv. 28	C.	—	200	200	200
6	27. vii. 28	S.	—	100	—	6
7	16. iv. 29	J. K.	0	—	1	—
8	18. vi. 29	F. R.	—	20	1	0
9	19. vi. 29	E. J.	—	Hs.	—	—
10	19. vi. 29	J. C.	—	Hs.	1	—
11	19. vi. 29	A. C.	—	0	4	12
12	19. vi. 29	M. J.	—	20	45	2
13	19. vi. 29	W. A.	—	20	—	—
14	26. vi. 29	K.	—	—	0	4
15	26. vi. 29	H.	—	20	20	6
16	26. vi. 29	R. B.	1	—	20	—
17	26. vi. 29	J. B.	—	20	20	20
18	19. vii. 29	B.	—	Hs.	—	—
19	23. vii. 29	S. McB.	—	Hs.	—	Hs.
20	23. vii. 29	C.	—	0	20	—
21	24. vii. 29	S. T.	20	Hs.	10	0
22	29. vii. 29	M. W.	0	3	0	0
23	10. viii. 29	M. S.	0	1	0	0
24	10. viii. 29	R.	15	—	20	—
25	29. viii. 29	M. M. D.	0	—	—	70
26	29. viii. 29	M. M.	20	—	—	200
27	29. viii. 29	M. D.	—	—	—	100
28	3. ix. 29	S. B.	—	160	—	150
29	21. ix. 29	S. M. Y.	0	0	—	1
30	21. ix. 29	M. D.	Hs.	Hs.	—	0
31	21. ix. 29	M. Morg.	0	2	—	34
32	21. ix. 29	A. R.	50	50	—	100
33	22. x. 29	M. H.	—	0	—	81
34	11. i. 30	S. H.	20	50	50	50
35	8. iv. 30	E. C.	24	Hs.	Hs.	Hs.
36	24. iv. 30	M. B.	Hs.	Hs.	Hs.	Hs.

Hs. = hundreds.

In our earlier tests the great delay in the appearance of *B. paratyphosus B* colonies on the bismuth media was not fully appreciated, and this fact no doubt explains some of our failures with the media. If the stools are stale and *B. proteus* is abundant this organism may interfere with the development of *B. paratyphosus B*. The bismuth media inhibit the growth of *B. pyocyaneus*, which sometimes renders difficult the isolation of *B. paratyphosus B* on the MacConkey or MacConkey brilliant green plates.

ISOLATION OF *B. TYPHOSUS* FROM SEWAGE.

In the experiments fresh crude screened sewage was taken in sterile bottles as it flowed in open channels to sedimentation tanks. In the majority of the tests the sewage was from the City of Belfast, but in a few it was obtained from Lisburn, a neighbouring town. On an average about ten plates containing the bismuth medium B were inoculated on their surface with about  $\frac{1}{4}$  or  $\frac{1}{2}$  c.c. of the sewage. The surface was allowed to dry and the plates were inverted. Occasionally the sewage was mixed with water and then incorporated with the medium, which was then poured out into the plates. After 24–36 hours in the incubator isolated black colonies were subcultured on to a modified Endo medium. This medium was prepared by dissolving 1 grm. of lactose and 1 grm. of saccharose in about 15 c.c. of boiling water, adding melted 3 per cent. nutrient agar up to a volume of 200 c.c., and then making a final addition of 0.5 c.c. of alcoholic solution of fuchsin (2 grm. in 60 c.c. alcohol) and of 3 c.c. of a 20 per cent. solution of sodium sulphite.

The addition of 0.5 or 1 c.c. of liquor bismuthi prevents any *Proteus* bacilli which are subcultured from forming a spreading growth over the plates. On the dry surface of this medium each black colony was planted over an area about the size of a sixpence. Often within 8 hours, and always within 15 hours, it was possible to determine whether the colonies consisted of fermenters or non-fermenters of the lactose and saccharose. The acid formers nearly all appeared to belong to a group of organisms to which Wilson (1928) has given the name *B. effluviæ*.

The agglutinability of the non-fermenting colonies was tested by removing a little of the growth on a platinum loop, and rubbing it up in a drop of a 1 in 60 dilution of a typhoid serum on a slide. The slide was then rocked to and fro over an electric lamp and the presence or absence of agglutination noted. If agglutination occurred, the colony from which the bacilli had been taken was subcultured into mannite broth containing Andrade's indicator, and then about an equal volume of melted agar (cooled to 50° C.) added. On incubation, if acid but no gas was formed, pure cultures were obtained from the colony, and their action on glucose, dulcitol, saccharose and lactose was studied. Cultures in peptone water were tested for indole formation. The identification was completed by seeing whether the organism was agglutinated to full titre by a typhoid-agglutinating serum. In a considerable number of cases the capacity of the organism to remove agglutinins from a typhoid serum was examined. The organisms were motile and did not liquefy gelatin or inspissated serum.

Planted on Fleming's "influenza" medium the *B. typhosus* causes no clearing of the haemoglobin derivatives, whereas a zone of clearing occurs around the saccharose and lactose fermenting colonies of *B. effluviæ*, and also with certain non-fermenters which probably are closely allied to the *B. effluviæ* group.



As we discarded the majority of the organisms which were agglutinated by a strong anti-typhoid serum and which were subsequently found to produce acid and gas in glucose shake cultures, it is very probable that we missed many *B. paratyphosus* B strains, especially where the cultures were made after 48 hours' incubation of the bismuth media plates.

In Table III the results of experiments carried out since 30. viii. 28, the date of a former report by one of us (Wilson, 1928), are summarised.

Table III.

No. of exp.	Origin of sewage	Date	Amount of sewage examined in c.c.	Black colonies tested	No. of sac-charose or lactose fermenters	No. of non-sac-charose or lactose fermenters	No. of typhoid colonies found
1	Belfast	30. iii. 28	4.5	93	77	16	0
2	Belfast	17. v. 28	7.5	97	67	30	11
3	Belfast	5. vi. 28	7.5	93	72	21	9
4	Lisburn	22. viii. 28	5.0	19	7	12	18
5	Belfast	3. ix. 28	5.0	150	150	0	0
6	Belfast	8. ix. 28	5.0	150	150	0	0
7	Belfast	20. ix. 28	4.0	10	9	1	1
8	Belfast	9. xi. 28	4.0	115	108	7	1
9	Belfast	21. xii. 28	6.0	119	98	21	7
10	Belfast	14. i. 29	6.0	83	45	38	33
11	Belfast	18. ii. 29	1.0	15	0	15	11
12	Belfast	8. iii. 29	2.0	52	38	14	4
13	Lisburn	8. v. 29	2.0	37	34	3	2
14	Belfast	30. vii. 29	6.0	69	53	16	5
15	Belfast	12. ix. 29	5.0	70	60	10	6
Total			70.5	1172	968	204	108

It is seen that 12 out of the 15 experiments resulted in the isolation of the *B. typhosus*. The total volume of the sewage examined was 70 c.c. and from it 108 typhoid bacilli were isolated. There is, of course, irregularity in the distribution of bacilli in the sewage but, on an average, there is at least one typhoid bacillus in every c.c. of the sewage taken at any time the whole year round. The failure of experiments 5 and 6 was due to the large numbers of black colonies consisting mainly of saccharose fermenters.

In July, August and September there is a seasonal multiplication of sulphite-reducing organisms in sewage and in water, and at this season the isolation of the *B. typhosus* is rendered more difficult. Wilson (1928) has referred to this, and it has also been noted by A. C. Houston (1928).

#### ISOLATION OF *B. PARATYPHOSUS* B FROM SEWAGE.

As already observed, no attempts to isolate *B. paratyphosus* B were made in the earlier experiments. It is not improbable that *B. paratyphosus* B is as widely disseminated in Belfast sewage as is *B. typhosus*. Here we will refer to two occasions on which it was isolated from Belfast sewage. Of the 14 non-fermenters of lactose and saccharose isolated from the sewage of 8. iii. 29, seven were agglutinated on a slide by typhoid serum. Of these seven, four proved to be *B. typhosus* and two *B. paratyphosus* B.

On 12. iii. 29 1 c.c. of the sewage of 8. iii. 29 was mixed with 10 c.c. of water and then mixed with an equal volume of double strength bismuth medium B and poured out into a plate. In all 15 c.c. of the sewage were utilised in this way, and 15 cultures of *B. paratyphosus* B were obtained.

The sample of Belfast sewage taken on 12. ix. 29 and which, as we have noted, yielded 10 black colonies consisting of non-fermenters of lactose and saccharose, and nine of these were agglutinated by a strong typhoid serum. Subsequent tests showed that six of the nine were typhoid bacilli, and that three were paratyphoid B bacilli.

#### VIABILITY OF *B. TYPHOSUS* IN SEWAGE.

On 5. vi. 28 four Winchester Quart bottles, labelled *A*, *B*, *C* and *D*, were taken of Belfast crude screened sewage.

We have already seen that from 7.5 c.c. of this sewage nine typhoid bacilli were cultivated. The sewage contained 500,000 *B. coli* and 3000 spores of *B. welchii* per c.c. The bottles were stoppered and kept at room temperature.

On 13. vi. 28 bottle *B* was shaken and 1 c.c. was planted over the surface of each of four plates containing the bismuth brilliant green medium. Seven black colonies were studied, and five proved to be typhoid bacilli.

On 15. vi. 28 one c.c. on the surface of each of four plates yielded eight black colonies, two of which proved to consist of typhoid bacilli. On the same date four plates were poured out after 1 c.c., 5 c.c., 5 c.c. and 5 c.c. of sewage had been added to the melted and cooled medium. Fifty black colonies were studied; 48 were lactose and saccharose fermenters and two proved to be typhoid bacilli.

On 18. vi. 28 and on 22. vi. 28 we were unsuccessful in isolating any typhoid bacilli.

On 23. vi. 28 ten c.c. of the sewage in bottle *B* were mixed with an equal volume of the bismuth brilliant green medium and poured into plates. Fourteen black colonies developed, six were non-fermenters of lactose and saccharose, and of these I gave all the usual cultural characters of the *B. typhosus*, and was agglutinated up to full titre. This bacillus had survived for 18 days, *i.e.* from 5. vi. 28 till 23. vi. 28.

On 26. vi. 28 seven plates were inoculated with bottle *B* sewage which had, during the latter experiments, been exposed to the air and fairly well aerated; no black colonies of any kind developed.

On 26. vi. 28 ten c.c. of sewage of 5. vi. 28 contained in bottle *D* which was stoppered and undisturbed were planted out; 52 colonies were studied, 31 being fermenters and 21 non-fermenters of saccharose and lactose. Nine of the 21 were agglutinated by a typhoid serum but seven formed acid and gas and only two proved to be typhoid bacilli. Those which were agglutinated in low titre by the typhoid serum but which formed acid and gas in glucose may have been paratyphoid bacilli, but this point was not determined.

On 28 vi. 28 the deposit in bottle *D* was stirred up and 10 c.c. of the

contents were planted out: 51 black colonies were examined, 20 being non-fermenters of lactose and saccharose, and of these two were agglutinated to full titre with a typhoid serum and gave the cultural characters of *B. typhosus*.

On 10. vii. 28 thirty c.c. from bottle *D* were examined with negative results, but, on 12. vii. five black colonies developed from inoculations made on that date, and one proved to be *B. typhosus*.

On 13. vii. 28 the top liquor was poured off from bottle *D* leaving 100 c.c. at the bottom containing most of the larger solids; this was mixed with 100 c.c. of sterile water and 10 c.c. amounts mixed with melted bismuth brilliant green medium *B*, and poured out into each of five plates; 20 black colonies were found, of which nine were non-fermenters of lactose and saccharose and of which one proved to be the typhoid bacillus. This bacillus produced acid but no gas in glucose, maltose and mannite, had no action on saccharose, lactose and dulcitol, formed no indole, and was agglutinated to full titre with an anti-typhoid serum. This result showed that there was at least one typhoid bacillus alive in the sewage after storage in bottle from 5. vi. till 13. vii. 28.

Further tests of the watery deposit in bottle *D* were made on 13. vii., 17. vii. and on 23. vii., but no typhoid bacilli were isolated. On 25. vii. supernatant liquor which smelled strongly of sulphuretted hydrogen was poured off from bottle *C* and 50 c.c. of the fluid at the bottom of the bottle were planted out in bismuth medium *B*; 36 black colonies developed and of these 29 were non-fermenters of lactose and saccharose but proved not to be typhoid bacilli.

From this experiment we may conclude that it is possible for the typhoid bacillus, which is in sewage under natural conditions, to survive for over 5 weeks (38 days) when kept in a Winchester Quart bottle at room temperature and exposed to light. Originally there were at least 500,000 *B. coli* present in each c.c. of the sewage, and there was no doubt a bacteriophage for the *B. typhosus* present in the sewage. We may observe that the bacilli seemed to disappear from the supernatant liquor and to survive in the sediment. Dodgson (1928) remarks: "An important consideration in connection with water directly polluted by crude sewage is the possibility of the presence of undisintegrated faeces, etc. Even if such material be merely particulate any typhoid bacilli which it may contain may be protected against adverse conditions to which they might be exposed if free in the water. Gaertner and Rubner and Ohlmüller have investigated this aspect of the question and claim to have shown that envelopment in particles of faeces definitely favours the survival of the bacilli." We have no doubt from our experiment that such is the case.

A second experiment showed that typhoid bacilli could be isolated from crude sewage at the end of 16 days. The sewage of 14. i. 29, of which 33 typhoid bacilli had been isolated, was re-examined on 30. i. 29, and of 52 black colonies examined seven proved to be non-fermenters of lactose and saccharose, and of these two consisted of typhoid bacilli.

VIABILITY OF *B. TYPHOSUS* AND *B. PARATYPHOSUS* B IN SEWAGE.

The sewage of 8. iii. 29 was poured out into 8 oz. bottles, which were filled and corked and kept at room temperature in the laboratory in a dark press, on the roof or in a Frigidaire cupboard.

Cultures were made from the deposit in these bottles on 19. iii. 29 on the surface of bismuth brilliant green plates. The results were: from the bottle at room temperature four typhoid bacilli and two paratyphoid B bacilli were isolated. The corresponding numbers for bottles in dark press and Frigidaire and on the roof were: 2 and 1, 1 and 3, 0 and 2 respectively.

On 29. iii. 29 seven plates were inoculated on the surface with deposit from bottle kept at room temperature, and resulted in the isolation of two paratyphoid but no typhoid bacilli. On the same day, 29. iii. 29, similar cultures were made from the deposit in bottles kept in dark press and resulted in the isolation of one paratyphoid bacillus but no typhoid bacilli.

In this experiment typhoid bacilli survived in considerable numbers from 8. iii. 29 till 19. iii. 29, *i.e.* 11 days. The paratyphoid bacilli survived equally well, and a few were found alive on 29. iii. 29, *i.e.* after 21 days.

The older work on the survival of typhoid bacilli in sewage consisted in adding large numbers of typhoid bacilli to sewage or in exposing bacilli contained in celloidin bags (Russell and Fuller, 1906) to the action of the sewage.

Dodgson quotes from MacConkey's experiment as follows: "That although *B. typhosus* (8,000,000 per 1 c.c. added) might disappear from crude sewage within six days, it might persist in similar sewage in significant numbers for 13 days (reduction from 200,000 to 1000 per c.c.). In the latter case the reduction in the *B. typhosus* population was in about the same ratio as that of the sewage organisms in general."

Russell and Fuller state that the survival period of *B. typhosus* subjected to the action of sewage is from 3 to 5 days.

We think that our work gives laboratory support for the possibility of the survival of *B. typhosus* in the outer world sufficiently long to render possible water-borne epidemics, the occurrence of which has been definitely established by epidemiological investigations, but for the possibility of which laboratory investigators could produce no evidence.

ATTEMPTS TO SUPPRESS *B. EFFLUVIEI*.

It was soon appreciated that, if the bismuth media could be modified in such a way as to inhibit the growth of organisms other than *B. typhosus* which formed black colonies on the plates and which we may consider as belonging to the group of *B. effluviei* (Wilson), the isolation of *B. typhosus* from sewage and water could be facilitated especially during July, August, and September when the organisms appear to show a seasonal increase in numbers. In faeces, as far as our experience indicates, *B. effluviei* is never present.

As the reduction of sulphites is probably connected with the respiration

of the bacteria and, as we had learned from Meyerhof's *Chemical Dynamics of Life Phenomena*, that narcotics inhibit the rate of oxidation, we were led to try the effects of the addition of certain narcotics to the bismuth media.

We may say at once that it was found possible in this way to inhibit the growth of certain strains of *B. effluviei* whilst allowing that of *B. typhosus*, but that in practice no great advantage occurred when we were dealing with the various strains met with in sewage.

In our experiments we made use of acetamide, urethane, chloral hydrate, barbitonum, chlorobutol, phenyl-urea, di-ethyl-urea, methyl-urea, di-ethyl-diphenyl-urea, acetone, chloroform, sulphonal and methyl-sulphonal.

The following concentrations of a few of these narcotics appeared to yield promising results for our purpose. If to 100 c.c. of agar the usual constituents of the bismuth media were added, it was found that the subsequent addition of 16 c.c. of a 16 per cent. watery solution of acetamide, of 0.7 c.c. of a 10 per cent. watery solution of chloral hydrate, 8 c.c. of a 10 per cent. solution of urethane, and 3 c.c. of a 5 per cent. alcoholic solution of barbitonum, did not prevent the development of *B. typhosus*, did inhibit some strains of *B. effluviei*, but in actual tests with sewage the modifications had no advantage over our standard media.

The results of our experiment carried out with sewage of 14. i. 29 are given in Table IV.

Table IV.

Medium	Amount of sewage c.c.	Total black colonies tested	Sac- charose fermenters	Non- saccharose fermenters	Typhoid bacilli isolated
Standard bismuth B	1	25	17	8	4
+ Acetamide	1	16	5	11	9
+ Urethane	1	28	12	16	11
+ Barbitonum	1	7	5	2	1
+ Di-ethyl-di- phenyl-urea	1	2	2	0	0
+ Chloral	1	15	4	11	8

Our experiments, which need not be given here, showed that narcotics showed a decided selective action on the growth of various micro-organisms—a result to be expected from their action on the surface of membranes and, in the case of bacteria, it is known that specific differences are in many cases associated with the chemical constitution of their capsules.

#### ISOLATION OF *B. TYPHOSUS* AND *B. PARATYPHOSUS* B FROM WATER.

Encouraged by Sir Alexander Houston's success in isolating the *B. typhosus* from Thames water by means of our media, we have commenced the testing of the River Lagan.

Here we shall report the results of our two first experiments. The technique was similar to that employed in the investigation of sewage, but to concentrate the bacteria 2.5 c.c. of a 10 per cent. solution of aluminium sulphate were added to each litre of water, the pH being adjusted to about 7. The precipitate

was allowed to settle, the supernatant water aspirated off and the lower layer containing about 100 c.c. was put into tubes and centrifuged. Finally the precipitate in the bottom of the centrifuge tubes was spread over the surface of the bismuth medium B, or was incorporated in its substance when in the liquid condition.

*Exp. 1. 24. ix. 29.* Four litres of water from the River Lagan were taken about 2 miles above Belfast. Forty-nine black colonies were examined; 26 were lactose-saccharose fermenters and 23 non-fermenters, but none proved to be the *B. typhosus*, but were organisms capable of liquefying gelatin and of digesting inspissated serum.

*Exp. 2. 7. x. 29.* Four litres of water were taken from Lagan Canal close to the entrance of a stream into which the effluent of Lisburn sewage works discharged. The sewage of Lisburn is treated on contact-beds and then on land, and eventually the effluent reaches a stream which, after a course of about 200 yards, enters the Lagan Canal. The sewage is well purified and does not cause secondary decomposition in the canal. The water in our sample looked like water, but it contained a considerable amount of effluent, as *B. coli* were present in 0.001 c.c. and there were 12 spores of *B. welchii* per c.c. From the aluminium coagulum from this water planted on or in the usual bismuth medium B, and in the same medium with the addition of chloral, a considerable number of black colonies developed. Ninety-three of these were studied: 74 fermented lactose and saccharose; 19 were non-fermenters and of these four proved to be typhoid bacilli and two were *B. paratyphosus* B.

#### IDENTIFICATION OF *B. TYPHOSUS* AND *B. PARATYPHOSUS* B.

For routine work we regarded a bacillus as the *B. typhosus* if it formed a characteristic dry black colony on our bismuth media, gave a clear growth on Endo's medium containing lactose and saccharose, formed acid and no gas in agar shake cultures containing mannite and glucose (Andrade's Indicator) and was agglutinated to full titre by an anti-typhoid serum. In most instances specimens were examined for absence of liquefaction of gelatin and absence of indole formation.

In a considerable number of the strains isolated we not only tested their agglutinability by a typhoid serum, but also by absorption tests proved that they were able to remove from the serum the agglutinins not only for themselves but also those for typical *B. typhosus*.

Similar tests were employed in the identification of the *B. paratyphosus* B. We satisfied ourselves that the bacilli failed to ferment lactose and saccharose and that they formed acid and gas in mannite and glucose, formed no indole, did not liquefy gelatin and were agglutinated to full titre by a specific serum. Absorption tests were also employed.

In the case of four typhoid strains and two paratyphoid B strains isolated from the Lagan Canal, rabbits were inoculated and their sera tested on the

homologous strains and on strains isolated from enteric fever patients. Absorption tests proved that genuine typhoid and paratyphoid bacilli B removed the agglutinins both for themselves and for the suspected organisms from the serum of the rabbits; also absorption of specific anti-typhoid and paratyphoid B sera with the strains removed the agglutinins for the strains and also those for genuine typhoid and paratyphoid bacilli. In the case of a few strains additional tests were employed.

In the differential medium of Jordan and Harmon (1928) containing sodium-potassium tartrate (Rochelle salts) 18 typhoid cultures were tested; all gave an acid reaction and of the 18, 5 were isolated from patients and 13 from sewage.

Thirty-three paratyphoid strains, 21 from patients and 11 from sewage, all gave an alkaline reaction, whilst a laboratory culture of *B. aertrycke* (Mutton) gave an acid reaction.

#### RHAMNOSE FERMENTATION.

According to Bitter, Weigmann and Habs (1926), when paratyphoid B cultures or *aertrycke* (Breslau) cultures are sown in a saline peptone solution containing 0.5 per cent. of rhamnose and, after a growth of 15 or 16 hours at 37° C., two drops of methyl red indicator (0.5 grm. methyl red dissolved in 100 c.c. of alcohol) are added to each culture, the paratyphoid bacilli will show a distinct yellow or orange colour, whereas the *aertrycke* (Breslau) will become distinctly red.

This we can confirm to a large extent from our observations as regards cultures of *B. paratyphosus* B recently isolated from cases of paratyphoid fever. In an experiment, where we employed 23 strains of *B. paratyphosus* B recently isolated from paratyphoid patients, 15 strains of *B. paratyphosus* B isolated from sewage, one old stock laboratory culture of *B. paratyphosus* B and one culture of *B. aertrycke*, it was found after 22 hours when the first observation was made that 12 of the cultures were acid in reaction, and that these consisted of the *aertrycke* culture, the old laboratory stock culture of *B. paratyphosus* B and 10 sewage strains of *B. paratyphosus* B; the 28 others including all the strains isolated from patients were alkaline. After 72 hours eight (including two sewage strains) became acid, and, after 96 hours, all gave the red reaction. We find that Andrade's indicator can show the changes more conveniently than the use of methyl red.

In ordinary peptone water containing rhamnose, the same differences in the rate of fermentation are manifested, but the changes in reaction occur more quickly.

In an experiment where rhamnose peptone water was inoculated with 20 paratyphoid B cultures recently isolated from patients, one old laboratory stock culture of *B. paratyphosus* B, one *aertrycke* bacillus, and five strains of *B. paratyphosus* B isolated from sewage, we found six tubes acid at the end of 16 hours' incubation at 37° C., and that most of the others showed slight

change of reaction at the end of 28 hours, going on to complete reddening at the end of 40 hours. The six cultures bringing about the rapid fermentation in this experiment consisted of our old stock culture of *B. paratyphosus* B, the *aertrycke* bacillus and four sewage paratyphoid bacilli.

These experiments would seem to indicate that, as regards fermentation of rhamnose, the majority of our sewage strains of *B. paratyphosus* B behaved more like the *aertrycke* bacillus than those of the strains of *B. paratyphosus* B we were finding in the stools of cases of paratyphoid fever. We may observe, however, that the 28 strains isolated from the River Lagan were slow fermenters and that it was in County Down, in which county the river rises, that most of our enteric fever patients resided.

That our sewage cultures were genuine *B. paratyphoid* B is probable from our agglutination and absorption experiments, even though we did not attempt a receptor analysis. Moreover, they behaved in Jordan and Harmon's medium like the *B. paratyphosus* B and not like *B. aertrycke*.

#### ISOLATION OF *B. ENTERITIDIS* GAERTNER FROM STOOLS.

On Wednesday, 16. x. 29, an outbreak of food poisoning occurred in Ballymena—a town about 30 miles distant from Belfast. The outbreak was not thoroughly investigated, but it is almost certain that the infection was conveyed by milk, and that the milk acquired the *B. enteritidis* Gaertner from a sick cow.

Through the kind services of Dr John Stuart (Ballymena) and Mr J. C. Loughridge F.R.C.S. (Belfast) we had an opportunity of examining 14 specimens of stools from 14 patients. The faeces of one patient (*D*) were taken on 18. x. 29, and were planted on MacConkey's medium containing brilliant green, and on our old standard bismuth brilliant green medium. On the MacConkey medium no members of the Salmonella group developed, but on the bismuth medium, after a delay of 60 hours, 200 colonies of *B. enteritidis* Gaertner appeared. On 21. x. 29 13 other stools were similarly tested with results shown in Table V.

Table V. Colonies of *B. enteritidis* Gaertner.

Patient's no.	MacConkey + brilliant green	On old standard bismuth brilliant green medium on fourth day
1	Negative	20
2	"	2
3	"	Negative
4	"	100
5	"	6
6	"	Negative
7	"	"
8	"	"
9	100	Several hundred
10	Several hundred	"
11	"	"
12	"	"
13	"	"



The 14 stools, therefore, yielded positive results ten times with the bismuth medium and five times with the MacConkey medium. The examination was made when many of the patients were convalescent about 5 days after the acute illness. In this outbreak, in addition to human beings, cats and dogs suffered. It is worthy of record that, in spite of the large number of bacilli in the stools of the patients, no secondary cases occurred. Immediately after the start of the outbreak the sick cow was slaughtered.

## PART II.

Numerous experiments were carried out using the sulphite and bismuth solutions in different proportions. It was found that it was possible to obtain black surface colonies of *B. typhosus* in a medium free from iron. This new medium will be referred to in this paper as the New Standard bismuth medium to distinguish it from the older iron-containing medium, which we shall designate Old Standard medium B.

### NEW STANDARD BISMUTH SULPHITE MEDIUM.

This medium is prepared in the usual way and contains: 100 c.c. nutrient agar, 2.5 c.c. of 20 per cent. glucose, 5 c.c. of 20 per cent. sodium sulphite, 2.5 c.c. liq. bismuthi, 0.5 gm. sodium phosphate (exsiccated), 0.5 c.c. of a 1 per cent. solution of brilliant green.

On this medium the *B. typhosus* forms black colonies, as does also the *B. paratyphosus* B, the latter growing better when only 3 c.c. of sodium sulphite instead of 5 c.c. are used.

The New Standard medium, if mixed with an equal volume of water containing a few typhoid bacilli and the mixture poured out into plates, allows the formation of black colonies in its depth as well as on its surface.

For the development of typhoid colonies in the depths of a tube or plate we have found the following formulae useful.

#### *For deep colonies in tubes.*

(1) 100 c.c. nutrient 3 per cent. agar, 10 c.c. glucose 20 per cent., 2 c.c. sulphite 20 per cent. (for plates use 3 c.c.), 3 c.c. liq. bismuthi, 1 c.c. 8 per cent. ferrous sulphate solution, 1 c.c. of a 1 per cent. solution of brilliant green.

(2) 100 c.c. nutrient 3 per cent. agar, 5 c.c. glucose 20 per cent., 1.5 c.c. sulphite 20 per cent., 3 c.c. liq. bismuthi, 0.5 c.c. of a 1 per cent. solution of brilliant green.

For the typhoid bacillus 2 c.c. of sodium sulphite with 2 c.c. of liq. bismuthi may be employed, for *paratyphosus* B, 2 c.c. of liq. bismuthi to 1 c.c. of sodium sulphite gives better results.

#### *For deep colonies in plates.*

100 c.c. nutrient agar 3 per cent., 2.5 c.c. glucose 20 per cent., 5 c.c. sulphite 20 per cent., 2.5 c.c. liq. bismuthi, 1 gm. exsiccated sodium phosphate,

0.5 c.c. of a 1 per cent. solution of brilliant green. The above is very suitable for *B. typhosus*, less suitable for *paratyphosus* B; for the latter use 2 c.c. sulphite and 3 c.c. liq. bismuthi.

#### STANDARDISATION OF THE MEDIA.

We have employed the media for over 3 years and never have had any trouble with them. Others would appear to have found the results inconstant. Buonomini (1929) found that the blackening of the colonies depended on the brand of bismuth citrate employed. We had employed bismuth citrate obtained from Baird and Tatlock, London, and from the British Drug Houses, Ltd. The secretary of the latter company has informed us that their bismuth citrate is prepared "by mixing bismuth subnitrate and citric acid with distilled water, and heating on a water bath with frequent stirring until a drop of the mixture yields a clear solution with ammonia: then adding water, allowing the salt to deposit, washing the precipitate so obtained until the washings are tasteless, and drying at a gentle heat." After the appearance of Buonomini's paper we obtained samples of bismuth citrate from Merck and from Kahlbaum: solutions of liq. bismuthi prepared in our usual way from these samples were compared with those that we were using. The appearance of the liquors differed, some being clear, some slightly turbid, but in our tests all gave satisfactory results—good black colonies.

For the guidance of those employing the medium for the first time, we would emphasise the importance of inoculating the plates with water containing a very few typhoid bacilli, so that discrete colonies may be obtained, as, where the growth is confluent, no darkening may occur.

As regards the solution of sulphite, it is a matter of indifference whether a 20 per cent. solution of sodium sulphite (exsiccated) or a 40 per cent. solution of sodium sulphite crystals is employed.

As regards the iron solution, we employed an 8 per cent. solution of ferrous sulphate crystals, but Buonomini prefers an 8 per cent. solution of ferrous ammonium sulphate  $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ .

It had been our practice to use Fairchild's peptone, and we always found it suitable for our media. We then tested Bacto peptone and found it equally good, but with Witte's peptone the colonies of typhoid did not come up well, and in many cases did not blacken. In the preparation of the media, therefore, Witte's peptone should be avoided.

For making nutrient agar, we employ 3 per cent. powdered agar No. 1 quality, 1 per cent. peptone (Fairchild's or Bacto), and 0.5 per cent. Lemco (Liebig's Extract of Meat). Ordinary Lemco does very well, but the special preparation Lab-Lemco is to be preferred.

We have used ordinary tap-water, but distilled water in many parts of the world may be necessary, as there may be salts in the water which interfere with the reduction process. We have found that the presence of 0.05 per cent. of potassium nitrate in the old standard medium prevents the development

of jet black colonies. The addition of brilliant green to the medium not only aids the inhibition and destruction of *B. coli* but promotes blackening. Blackening is also promoted by the addition of 2 c.c. of a 5 per cent. solution of tartar emetic to every 100 c.c. of the Old Standard medium.

We have already emphasised the importance of the addition of 1 gm. of exsiccated sodium phosphate dissolved in 10 c.c. of distilled water to each 100 c.c. of the Old Standard medium.

#### STOCK BISMUTH SULPHITE MIXTURES.

Experiments made to ascertain if it were possible to get equally good results with stock solutions containing a mixture of the constituents, instead of adding them separately, showed that this was the case.

For example, the addition of 16 c.c. of the following mixture to 100 c.c. of hot nutrient agar plus 5 c.c. of a 20 per cent. solution of glucose, provides a medium yielding excellent black surface colonies of *B. typhosus*: 100 c.c. sodium sulphite 20 per cent., 50 c.c. liq. bismuthi, 10 c.c. of an 8 per cent. solution of ferrous sulphate crystals and 10 gm. of exsiccated sodium phosphate. These reagents boiled together give a mixture which has been found to remain active for over a month.

The mixture for the New Standard medium is made by boiling together 100 c.c. of a 20 per cent. sodium sulphite solution with 50 c.c. liq. bismuthi and 10.5 gm. of sodium phosphate (exsiccated). Of this mixture (warmed if necessary in cold weather to secure solution of the phosphate) 7 c.c. are added to 100 c.c. hot nutrient agar plus 2.5 c.c. of a 20 per cent. solution of glucose. In the case of both, the media are completed by the addition of 0.5 c.c. of a 1 per cent. solution of brilliant green.

#### SELECTIVE ACTION OF ALCOHOLS.

Experiments were made with different amounts of the mixtures in glucose and lactose agar, and the growth thereon of different intestinal bacteria was studied.

In the course of the investigation use was made of an alcoholic solution of phenol red as an indicator. It was found that the addition of the alcoholic solution of phenol red suppressed the growth of certain sewage organisms which developed on the bismuth plates, and which rendered the isolation of typhoid bacilli more difficult. Further work showed that it was the alcohol and not the phenol red which tended to suppress the sewage organisms, whilst allowing the development of the *B. typhosus*.

From numerous tests we conclude that, for the isolation of *B. typhosus* from sewage, the addition of 2 c.c. of absolute alcohol to every 100 c.c. of nutrient agar in the Old Standard bismuth sulphite brilliant green medium, and of 4 c.c. or 5 c.c. to the New Standard brilliant green medium is an advantage.

We have also studied the action of methyl alcohol, propyl alcohol and amyl alcohol, and recommend the addition of 1 c.c. and 1.6 c.c. of propyl alcohol to the Old and the New Standard bismuth sulphite brilliant green media respectively.

The addition of ethyl alcohol tends to prevent the development of *B. paratyphosus* B in the New Standard medium, but this does not result from the addition of propyl alcohol.

For the development of *B. typhosus* in the depth of the medium we recommend the following medium to be mixed when fluid with an equal volume of water containing typhoid bacilli and to be poured out into Petri dishes: 100 c.c. nutrient agar, 2.5 c.c. glucose (20 per cent.), 7 c.c. from a mixture consisting of 100 c.c. of 20 per cent. sodium sulphite (anhydrous), 50 c.c. liq. bismuthi and 10.5 gm. sodium phosphate (exsiccated), 1 c.c. of a 1 per cent. solution of brilliant green and then 3.5 c.c. of propyl alcohol or 6 or 7 c.c. of absolute ethyl alcohol.

The propyl alcohol is suitable either for *B. typhosus* or *B. paratyphosus* B, and the ethyl alcohol for *B. typhosus* only.

The addition of the alcohols tend to suppress the growth of the *B. effluviei*, dark colonies of which are especially abundant in sewage in July and August.

#### SELECTIVE ACTION OF MERCURY SALTS.

As *B. proteus* grew quite as well as *B. typhosus* on the bismuth media, an effort was made to discover a modification of the medium which would allow the growth of *B. typhosus* and suppress that of various strains of the *proteus* group. In this connection experiments were made with salts of antimony, copper, silver and mercury. It was found that approximately 1 in 400,000 of perchloride of mercury in the bismuth brilliant green media suppressed to a large extent the growth of many *proteus* strains, whilst allowing the growth of *B. typhosus*.

The addition of 4 c.c. of a 1 in 10,000 watery solution of perchloride of mercury to a medium which has been prepared from 100 c.c. nutrient agar, 2.5 c.c. glucose 20 per cent., 7 c.c. of the mixture (100 c.c. sod. sulphite 20 per cent., 50 c.c. liquor bismuthi, 10.5 gm. sodium phosphate exsiccated), 0.5 c.c. of a 1 per cent. solution of brilliant green afforded a substrate on the surface of which, when poured out in Petri dishes, colonies of *B. typhosus* developed in normal fashion, whilst those of most strains of the *proteus* group failed to appear. It was also found that the mercury solution could be used in conjunction with alcohol, and that, in some instances, the above medium was improved by the addition of 4 c.c. of absolute alcohol.

After our preliminary experiments, we proceeded to try out our new media by inoculating them with sewage.

*B. typhosus and B. paratyphosus B*ISOLATION OF *B. TYPHOSUS* OR *B. PARATYPHOSUS* B FROM SEWAGE  
BY MEANS OF VARIOUS MEDIA.

## I. Old Standard glucose bismuth sulphite brilliant green medium.

(a)	(b)	(c)	(d)	(e)	(f)	(g)
Sewage	Date	Amount of sewage in c.c.	No. of black colonies	Fermenters of lactose and sac- charose	Non- fermenters of lactose and sac- charose	No. of typhoid or para- typhoid isolated
Belfast	15. xii. 29	1	30	24	6	3 typhoid
"	12. ii. 30	1	13	5	8	5 paratyphoid
"	24. ii. 30	1	16	2	14	13 typhoid
"	8. iii. 30	1	5	4	1	0
"	28. iv. 30	1	20	20	0	0
"	19. v. 30	1	11	9	2	0
"	29. vii. 30	1	9	9	0	0

## II. Old Standard medium plus 2 c.c. of absolute alcohol to every 100 c.c. nutrient agar.

(a)	(b)	(c)	(d)	(e)	(f)	(g)
Belfast	8. iii. 30	1	1.4	11	3	3 typhoid

## III. New Standard medium.

(a)	(b)	(c)	(d)	(e)	(f)	(g)
Belfast	15. xii. 29	1	6	6	6	5 typhoid
"	12. ii. 30	1	20	15	5	1 typhoid and 3 paratyphoid B
"	24. ii. 30	1	20	7	13	10 typhoid
"	6. vi. 30	1	5	5	0	0
"	29. vii. 30	1	3	3	0	0

IV. New Standard containing for every 100 c.c. of nutrient agar: (1) 3 c.c. absolute alcohol; (2) 4 c.c. absolute alcohol; (3) 5 c.c. absolute alcohol; (4) 4 or 5 c.c. of  $\text{HgCl}_2$  1 in 10,000; (5) 3 c.c. absolute alcohol plus 4 c.c. of  $\text{HgCl}_2$  1 in 10,000; (6) 4 c.c. absolute alcohol plus 4 c.c. of  $\text{HgCl}_2$  1 in 10,000.

(a)	(b)	(c)	(d)	(e)	(f)	(g)
(1) Belfast	6. vi. 30	1	4	0	4	3 typhoid
(2) "	28. iv. 30	1	5	1	4	3 "
"	6. vi. 30	1	10	0	10	6 "
"	29. vii. 30	1	6	3	3	3 "
(3) "	12. ii. 30	1	13	1	12	10 "
"	28. iv. 30	1	0	0	0	0
"	29. vii. 30	1	5	3	2	2 "
(4) "	12. ii. 30	1	6	5	1	1 paratyphoid
(5) "	6. vi. 30	1	6	1	5	4 typhoid
(6) "	6. vi. 30	1	5	0	5	2 "
"	29. vii. 30	1	11	11	0	0

*Deep colonies.*

The New Standard medium was mixed with an equal volume of water inoculated with 1 c.c. of sewage and poured out into plates. By this technique the sewage of 15. xii. 29 yielded several colonies of *paratyphosus* B. The same medium plus 3.5 c.c. of propyl alcohol per 100 c.c. of nutrient agar with sewage

of 8. iii. 30 which was employed on 17. iii. 30 yielded four black colonies, two of which proved to be typhoid and one of which was *paratyphosus* B.

A medium consisting of 100 c.c. nutrient agar, 5 c.c. glucose, 1 c.c. liq. bismuthi, 1 c.c. sulphite and 0.5 c.c. brilliant green mixed with an equal volume of water and poured into tubes, yielded with 1 c.c. of sewage of 15. xii. 29 numerous black colonies: 17 were examined, and of four which failed to ferment lactose and saccharose, two proved to be *paratyphosus* B.

### PART III.

#### A FLUID ENRICHMENT BISMUTH MEDIUM.

We have employed fluid enrichment media containing sulphite, bismuth and brilliant green, and have succeeded in securing multiplication of typhoid bacilli and, at the same time, the inhibition or even destruction of *B. coli*. It is only recently that we have obtained encouraging results, as in our earlier experiments the *B. typhosus* was very frequently outgrown by various strains of the *proteus* and *alcaligenes* groups. Now by the addition of alcohol and perchloride of mercury and possibly also by the use of mannite instead of glucose it is possible to suppress these strains.

As a basis nutrient broth is employed, and for the formulae suggested by us it will be found necessary to use Fairchild's peptone and not Bacto or Witte's peptone. No doubt for these two brands formulae could be devised, but so far we prefer to use Fairchild's, as better growth of the *B. typhosus* occurs in broth made from it when our reagents and especially alcohol and perchloride of mercury are added.

The broth is made in the ordinary way and is filtered through filter paper to render it quite clear. It contains: distilled water 1000 c.c., Fairchild's peptone 10 grm., Lab-Lemco 5 grm., and its reaction is made faintly alkaline to litmus paper.

To the broth mannite is added to form a 0.5 per cent. solution. The enrichment medium is made by taking 100 c.c. mannite broth, adding to it 6 c.c. of a 20 per cent. solution of sodium sulphite (exsiccated) and 0.2 liq. bismuthi and by heating the mixture to the boiling point. A flocculent precipitate separates out and should be shaken through the mixture. Finally 0.5 c.c. of a 1 per cent. watery solution of brilliant green is added.

We may mention that in the preparation of the liquor bismuthi we have employed various brands of bismuth citrate, e.g. Baird and Tatlock's (London), British Drug Houses, Merck and Kahlbaum, and have found them all satisfactory.

This medium we shall designate Standard bismuth broth (*A*), and of it we have made several modifications by the addition to every 100 c.c. of 1 c.c. of absolute ethyl alcohol (*B*), 1 c.c. of propyl alcohol (*C*), 2 c.c. of methyl alcohol (*D*), 1 c.c. absolute alcohol plus 4 c.c. of 1 in 10,000 solution of perchloride of mercury in distilled water (*E*).

ISOLATION OF *B. TYPHOSUS* FROM STOOLS BY BISMUTH FLUID  
ENRICHMENT MEDIA.

Table VI.

No.	Designation	Date	Standard broth				
			A	B	C	D	E
1	H.	12. iv. 30	+	+	-	...	...
2	J.	12. iv. 30	+	+	+	...	...
3	M. B.	22. iv. 30	+	+	+	+	...
4	C. M.	18. iv. 30	+	-	-	-	...
5	Ma. Bi.	9. v. 30	+	-	-	-	...
6	M. Bi.	9. v. 30	+	-	-	-	...
7	P. R.	24. v. 30	+	...	...	...	+
8	N. P.	27. v. 30	+	...	...	...	+
9	D. B.	29. v. 30	+	...	...	...	+
10	A. McI.	14. vi. 30	...	...	...	...	+
11	F. McI.	14. vi. 30	...	...	...	...	+
12	M.	16. vi. 30	+	...	...	...	+
13	T. R.	1. vii. 30	+	...	...	...	+
14	M. R.	1. vii. 30	+	...	...	...	+
15	M. S.	1. vii. 30	+	...	...	...	+
16	McE.	26. vii. 30	+	...	...	...	+
17	E. I.	19. viii. 30	+	...	...	...	+

From Table VI we see that the stools of 17 typhoid fever patients have been tested in the enrichment media and that 15 were tested in the Standard broth with 15 successes. Eleven were tested in the Standard broth containing in every 100 c.c. 1 c.c. of absolute alcohol, and 4 c.c. of a 1 in 10,000  $\text{HgCl}_2$  solution and in all 11 cases *B. typhosus* was isolated.

Subcultures from the broth tubes were made on to plates containing the Old Standard B medium and on to MacConkey plates. In this connection it is advisable to use MacConkey plates instead of Endo plates, as on the latter the bacilli are liable to be inagglutinable.

The use of alcohol and perchloride of mercury in the medium tends to the destruction of *proteus* strains. We may add that, in many cases, subcultures taken at intervals of 24 hours, 48 hours and 60 hours showed that there was a considerable multiplication of *B. typhosus* in the medium, and that the *B. coli* was not only inhibited in its growth, but was actually killed. Frequently on the MacConkey plates the subcultures afforded a pure growth of *B. typhosus*.

ISOLATION OF *B. TYPHOSUS* FROM SEWAGE BY MEANS OF  
STANDARD BISMUTH BROTH.

For this purpose tubes containing 25 c.c. of the broths were inoculated with 0.1 and 0.5 c.c. of the sewage. The results are given in Table VII.

Table VII.

Sewage	Deposit of sewage of 21. iv. 30	Date	Standard bismuth broth				
			A	B	C	D	E
Belfast		18. v. 30	-	...	...	...	+
"		19. v. 30	-	-	...	...	+
"		28. v. 30	...	...	...	...	+
"		6. vi. 30	...	...	...	...	-
"		29. vii. 30	-	...	...	...	-

It is, therefore, possible by means of a fluid bismuth sulphite enrichment medium to cultivate *B. typhosus* from sewage; whether as good results with this method could be obtained as with the Old and New Standard media containing alcohol, could only be shown by further investigations.

For the isolation of *B. typhosus* from faeces it is probable that where very few bacilli are present as in the case of some "carriers" and convalescents, the use of the fluid medium has advantages. At any rate on four occasions we were successful in demonstrating the presence of *B. typhosus* by its means where failure had occurred with the solid media.

#### DISCUSSION.

At the present stage it would be premature to attempt to explain the action of our bismuth sulphite media. One may hazard a guess that the inhibition of the growth of *B. coli* is concerned with the respiration of the bacillus. In fluid media and in deep cultures in solid media the growth of *B. typhosus* is best marked close to the surface. It would seem that our reagents interfere with the growth of *B. typhosus* unless a certain amount of oxygen is present. It is not improbable that the selective action on the growth of bacteria is determined by the varying permeability of the outer membrane of different species.

Whatever the explanation may be, it is an undoubted fact that a certain bismuth sulphite combination can kill *B. coli* and allow *B. typhosus* to grow, and this observation may find an application in the field of Chemotherapy.

It is possible that, along these lines, a substance may be found which would allow the growth of normal cells in the body, and at the same time inhibit the growth and destroy the cells of a malignant tumour, or those of a parasitic organism.

#### SUMMARY.

##### PART I.

1. By means of the Old Standard bismuth sulphite medium *B. typhosus* was cultivated 44 times out of 50 examinations of the stools of 50 typhoid fever patients and convalescents.

2. By means of the same medium *B. paratyphosus* B was cultivated 22 times out of 27 examinations of the stools of 27 different cases of paratyphoid fever.

3. In 13 examinations of Belfast sewage in different months during 1928 and 1929, by means of the Old Standard sulphite media, *B. typhosus* was isolated on 10 occasions. In two examinations of Lisburn sewage *B. typhosus* was isolated. Although no special search for *B. paratyphosus* B was undertaken, this organism was isolated on two occasions from Belfast sewage. The viability of *B. typhosus* and *B. paratyphosus* B in sewage is longer than was suggested by the work of previous observers. It has been possible to cultivate *B. typhosus* and *B. paratyphosus* B from the deposit of sewage stored in a



bottle at room temperature for 3 weeks. On one occasion *B. typhosus* was found alive at the end of 5 weeks.

4. On two occasions water from the River Lagan was examined, and on one of these *B. typhosus* and *B. paratyphosus B* were cultivated.

5. For the isolation of the *B. enteritidis* Gaertner the media gave good results in the examination of faeces from cases of food-poisoning.

## PART II.

6. Instead of adding the reagents separately in the preparation of the media, it has been shown that stock solutions containing sodium sulphite, liq. bismuthi, ferrous sulphate and sodium phosphate can be employed.

7. A New Standard bismuth medium is described. It is prepared by adding to 100 c.c. of hot 3 per cent. nutrient agar, 2.5 c.c. of a 20 per cent. solution of glucose and 7 c.c. of a mixture previously boiled consisting of 100 c.c. sodium sulphite (anhydrous) 20 per cent. solution, 50 c.c. liq. bismuthi, and 10.5 grm. of sodium phosphate (exsiccated). The medium is completed by the addition of 0.5 c.c. of a 1 per cent. solution of brilliant green.

8. The bismuth media are rendered still more selective for the isolation of *B. typhosus* from sewage by the addition of 2 c.c. of absolute alcohol to 100 c.c. of the Old Standard medium, and of 4 or 5 c.c. of absolute alcohol to the New Standard. For the isolation of *B. paratyphosus B* 2 c.c. of propyl alcohol should be employed in the New Standard medium.

9. From December 1929 to August 1930, seven specimens of Belfast sewage have been examined at monthly intervals, and in all cases *B. typhosus* has been isolated by means of the Standard media or of their modifications. On four occasions *B. paratyphosus B* was also isolated. The 10 successes out of 13 attempts recorded in Part I, the seven out of seven recorded in Part II together with the four out of four already reported in 1928, making 21 in all, show the almost constant presence of *B. typhosus* over a period of 3 years in Belfast sewage. 1 c.c. of the sewage as a rule contains at least one typhoid bacillus. Though not specially looked for, the *B. paratyphosus B* was isolated on six occasions.

## PART III.

10. In the preparation of the bismuth media Witte's peptone should not be employed, but Bacto or Fairchild's.

11. The addition of 4 c.c. of a 1 in 10,000 solution of perchloride of mercury to every 100 c.c. of the bismuth media tends to inhibit the growth of *proteus* strains and certain other organisms which develop from sewage, and whose presence interferes with the isolation of *B. typhosus*.

12. A fluid enrichment bismuth medium is described consisting of 100 c.c. mannite broth (Fairchild's peptone), 6 c.c. sodium sulphite 20 per cent. solution, 0.2 c.c. liq. bismuthi. To the boiled mixture 0.5 c.c. of a 1 per cent. solution of brilliant green is added. A useful modification of this medium is secured by the addition to every 100 c.c. of the mannite broth of 1 c.c. absolute

alcohol and 4 c.c. of a 1 in 10,000 solution of perchloride of mercury. By means of these fluid media the *B. typhosus* has been cultivated 17 times out of 17 stools from typhoid fever patients, and three times out of five specimens of sewage.

## REFERENCES.

- ADAMS, B. (1928). *The Medical Officer*, **39**, 185.  
 ALLISON, V. D. (1927-8). *Rep. of Lab. of Metropolitan Asylums' Board*. London.  
 BEGBIE, R. S. and GIBSON, H. J. (1930). *Brit. Med. J.* ii, 55.  
 BITTER, WEIGMANN and HABS (1926). *Münch. med. Wochenschr.* p. 940.  
 BUONOMINI, G. (1929). *Giorn. di Batter. e Immun.* **4**. Reprint.  
 D'ANTONA, L. (1929). *Ibid.* p. 323. Reprint.  
 DODGSON, R. W. (1928). Report on Mussel Purification. *Min. Agric. and Fish., Fishery Investig. Series II*, **10**, 193.  
 FRANCO, E. (1929). *Giorn. di Batter. e Immun.* **4**. Reprint.  
 GRAY, J. D. A. (1929). *Brit. Med. J.* i, 142.  
 HASHIMOTO, K. (1927). *Centralbl. f. Bakt. Orig.* **103**, 1.  
 HOUSTON, A. C. (1928). *Metropolitan Water Board, Twenty-third Annual Report*.  
 — (1929). *Ibid. Twenty-fourth Annual Report*.  
 JORDAN, E. O. and HARMON, P. H. (1928). *J. Infect. Dis.* **42**, 238.  
 MAZZETTI, G. (1928). *R. Accad. dei Fisiocritici di Siena*. Comunicaz. del 25. v. 28. Reprint.  
 NISSELE (1916). *Deutsche med. Wochenschr.* **42**.  
 RUSSELL, H. L. and FULLER, C. A. (1906). *J. Infect. Dis.* Supplement No. 2, p. 40.  
 SMITH, R. P. (1923). *J. Path. and Bact.* **26**, 122.  
 VIGNATI, J. (1928). *Centralbl. f. Bakt. Orig.* **107**, 54.  
 WILSON, W. J. and BLAIR, E. M. McV. (1927). *J. Hygiene*, **26**, 374.  
 WILSON, W. J. (23. vi. 1928). *Brit. Med. J.* i, 1061.  
 — (1928). *Public Health Congress*. London, Nov. 1928.

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## LETHARGIC ENCEPHALITIS: THE GLASGOW EPIDEMIC OF 1923.

ITS INCIDENCE AND CONSEQUENCES, FROM  
THE POINT OF VIEW OF PUBLIC HEALTH.

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### INTRODUCTION.

EPIDEMIC encephalitis made its appearance in Glasgow in 1918, although it was only in 1919 that its prevalence was such as to lead to its recognition. It recurred annually till 1924, and, during this period, the change in the clinical features from year to year suggested that it had passed through an evolutionary phase. While ocular symptoms were probably the features most common to all epidemics, prolonged lethargy and somnolence characterised the earlier cases; choreiform and myoclonic movements were more prevalent in 1920 and in 1921, while in 1924 there appeared for the first time a considerable proportion of cases with spinal signs and symptoms, which simulated acute attacks of insular sclerosis.

When the epidemic of 1923 began the medical profession in Glasgow was well prepared for its diagnosis. Doctors were supplied by the Medical Officer of Health with a memorandum in which were set forth the manifestations of the disease, which had been noted in the previous epidemics. Provision was made for notification and for hospital treatment, and, in this way, it was possible to form a fairly accurate estimate of the extent and character of the outbreak.

The following study is concerned with the cases of the 1923 epidemic, and with these only in a general and limited sense. An attempt is made to present a general view of the course of the disease, according as it terminated in early recovery in some cases, in apparent convalescence in others, or as, in the majority, it followed a protracted course along diverging lines in the form of characteristic sequelae. To obtain the purpose of the review, it has been necessary to confine the description of the sequelae to outlines of the main neurological and psychological features. So varied were the phenomena as a whole, and so fleeting many of the most prominent pathological signs, that it is expedient, in describing the general course of the disease as an epidemic, to confine attention to the hall-marks in which its identity is disclosed.

The data employed in the analysis have been obtained from several sources.

The original list of all the cases notified to the Medical Officer of Health, and accepted after observation as possible cases of acute encephalitis, was used as a basis. Along with this list, I have had the notes from hospital

records, as well as the private notes belonging to Dr I. Mackenzie, referring to the initial illness (Table I).

A year after the epidemic Dr Mackenzie examined all the cases, and I have used the results of his observations as a basis of the review of the condition of the cases in the spring of 1924. In the construction of this review I have gone over all the earlier hospital records, and have supplemented the information in Dr Mackenzie's records by further data, elicited from the patients and their friends (Table II).

This reconstructive review of the position, as it was in 1924, has been taken as a basis for the estimate of the later effects, as observed four years later, that is in the spring of 1928 (Table III).

In the course of the investigation of spring 1928, all the patients, reported and accepted as suffering from epidemic encephalitis in the spring of 1923, have been accounted for. All those surviving, with the exception of two, whose relatives were interviewed, have been examined. I am obliged to the superintendents of the various hospitals and asylums, in which the patients have been resident, for access to the records, to Prof. Leonard Findlay for notes on children, the course of whose subsequent history has been followed, and to Dr I. Mackenzie for the use of his records, and for his assistance and suggestions in my own examination of the patients and in the interpretation of the varied courses which their diseases have followed. In this way it has been sought to delineate the course of a phase of the epidemic, and so, by observing the character of the scar, which its imprint on the populace has produced, to provide a standard of reference by which this form of encephalitis may be compared with polio-encephalitis on the one hand, and, on the other, with the type which follows in the wake of influenza.

It is necessary to make a reservation in claiming for the description a representative character; for, as suggested already, the phase of 1923, while typical for the disease as a whole, differed in some respects from the other annual visitations; it did not bring the profound and prolonged somnolence, as in victims of the earlier phase, and there was an absence, from the series, of cases simulating disseminated sclerosis, such as characterised the phase of 1924. It has, however, the advantage that practically all the cases, which occurred in that period, must be included, and that it has been possible to follow their fate for a period of 5 years.

Table I comprises all the cases (70) of the original list, which were notified to the Medical Officer of Health in 1923 as possible cases of encephalitis lethargica, and includes a summary of the symptoms present during the initial phase of the illness.

#### MANIFESTATIONS OF LETHARGIC ENCEPHALITIS.

With the appearance of this unusual disease the physician had no conception of the gravity of the malady before him; he had not the slightest notion of the varied forms of clinical manifestations that were to present themselves,

Table I.

Case no.	Age at onset	Lethargy	Insomnia	Eye phenomena	Fever	C.S.F.	Tremors and twitches	Paresis	Reflexes	Neuralgias	Giddiness	Speech defect	Respiratory disturbance	Vomiting	Salivation	Classification according to condition in Spring 1928
1	8	-	-	-	-	+	+	-	+	-	-	-	-	-	-	Perversion of conduct
2	11	-	+	+	-	+	+	-	-	+	-	-	-	-	-	Died
3	11	+	-	-	-	+	+	+	+	-	+	-	-	-	-	Died
4	28	+	+	+	-	+	+	+	-	-	-	-	-	-	-	Parkinsonian (A)
5	24	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Eliminated
6	33	-	+	-	+	-	+	-	+	+	-	-	-	-	-	Died
7	47	+	-	+	+	+	+	+	+	-	-	-	-	-	-	Recovery complete
8	10	-	+	+	+	+	+	+	+	+	-	-	-	-	-	Parkinsonian (A)
9	7	-	+	+	-	-	+	-	-	+	-	+	-	-	-	Recovery incomplete (B)
10	19	-	+	+	+	-	+	+	-	-	-	-	-	+	-	Recovery incomplete (C)
11	24	-	+	+	-	-	+	-	-	+	-	-	-	-	-	Parkinsonian (A)
12	12	+	-	-	+	+	-	-	-	+	-	-	-	-	-	Recovery incomplete (E)
13	26	+	-	+	-	+	-	-	+	+	+	-	-	-	-	Recovery incomplete (C)
14	27	+	-	-	+	+	+	-	-	+	-	-	-	-	-	Recovery incomplete (C)
15	11	-	+	+	+	+	+	-	-	+	-	-	+	-	-	Recovery incomplete (E)
16	7	-	+	-	+	+	+	-	+	-	-	+	-	-	-	Parkinsonian (B)
17	7	+	-	+	+	-	+	-	-	-	-	-	-	-	-	Parkinsonian (B)
18	19	+	-	+	-	-	+	-	-	+	-	-	-	-	-	Recovery incomplete (C)
19	9	-	+	-	-	-	+	-	-	+	-	-	-	+	-	Perversion of conduct
20	13	+	-	-	-	+	+	+	+	+	-	+	-	-	-	Parkinsonian (B)
21	56	-	+	+	+	-	+	-	-	+	-	-	-	-	-	Died
22	16	+	-	+	-	-	-	+	-	+	+	-	-	-	-	Recovery incomplete (C)
23	23	+	+	+	-	-	+	-	+	+	+	-	-	-	-	Recovery incomplete (C)
24	23	+	+	+	+	-	+	+	+	+	-	-	-	-	-	Parkinsonian (A)
25	11	-	+	+	-	+	+	-	+	+	-	+	-	+	-	Parkinsonian (A)
26	1 $\frac{1}{2}$	-	+	+	-	-	-	-	-	-	-	-	-	-	-	Recovery incomplete (A)
27	8	+	+	-	+	+	+	+	-	-	-	-	-	-	-	Died
28	12	+	-	+	+	-	+	-	+	+	+	-	-	-	-	Recovery complete
29	12	+	-	+	+	+	+	+	+	+	+	+	-	+	-	Recovery incomplete (C)
30	15	+	-	+	+	-	-	+	+	+	+	-	-	-	-	Recovery incomplete (C)
31	40	+	+	+	-	-	+	+	-	+	-	-	+	-	-	Parkinsonian (A)
32	19	+	-	+	-	+	+	-	+	+	-	-	-	-	-	Recovery complete
33	14	-	+	+	-	+	+	-	+	+	-	-	-	-	-	Recovery incomplete (C)
34	27	-	+	+	+	+	-	-	-	-	-	-	-	+	-	Died
35	81	...	...	...	...	...	...	...	...	...	...	...	...	...	...	Eliminated
36	8	+	-	-	+	-	+	-	+	-	-	-	-	-	-	Recovery incomplete (A)
37	13	+	+	+	+	-	+	+	-	+	-	+	-	-	-	Parkinsonian (A)
38	12	-	+	+	-	-	+	-	-	+	-	-	-	-	-	Parkinsonian (A)
39	53	+	-	+	+	+	+	+	+	+	-	+	-	-	-	Died
40	13	-	+	+	+	+	+	-	-	+	-	-	-	+	-	Died
41	15	+	-	-	-	+	+	-	-	+	+	-	-	-	-	Recovery complete
42	2	+	-	-	+	+	+	+	-	+	-	-	-	+	-	Recovery complete
43	50	+	-	+	+	+	+	-	-	+	+	-	-	-	-	Parkinsonian (A)
44	22	+	-	-	+	-	+	-	+	+	+	+	+	+	-	Parkinsonian (A)
45	20	+	-	+	+	+	+	-	+	-	-	-	-	-	-	Died
46	9	-	+	+	-	-	+	+	+	+	-	-	-	-	-	Recovery incomplete (C)
47	4	+	-	+	+	-	+	-	+	-	-	-	-	-	-	Died
48	37	...	...	...	...	...	...	...	...	...	...	...	...	...	...	Eliminated
49	26	-	+	+	+	-	+	+	+	+	-	+	-	-	-	Recovery incomplete (C)
50	43	+	-	+	+	-	+	+	+	+	-	+	-	-	-	Died
51	7	+	-	+	-	+	+	-	+	+	-	+	-	-	-	Died
52	13	+	+	+	+	+	+	-	+	+	+	-	-	+	-	Parkinsonian (B)
53	5	+	-	+	+	-	+	+	+	+	-	-	-	-	-	Died
54	10	+	-	+	-	+	-	+	-	+	-	+	+	-	-	Parkinsonian (B)
55	19	-	+	+	+	-	+	-	-	-	-	-	-	-	-	Died
56	30	+	-	+	-	-	+	-	+	+	-	-	-	+	-	Recovery complete
57	19	-	+	+	+	-	+	-	-	+	-	-	-	-	-	Died
58	26	...	...	...	...	...	...	...	...	...	...	...	...	...	...	Eliminated
59	11	-	+	+	+	-	-	-	-	+	+	-	-	+	-	Parkinsonian (B)
60	5	-	+	-	+	+	+	+	-	-	-	-	-	-	-	Recovery incomplete (B)
61	48	+	-	+	+	+	+	-	+	+	-	-	-	+	-	Recovery incomplete (C)
62	23	+	-	+	+	-	+	-	+	+	-	-	-	-	-	Recovery complete
63	48	-	-	+	+	-	+	+	-	+	-	-	-	-	-	Died
64	5	-	+	-	+	-	+	-	-	-	-	+	-	-	-	Parkinsonian (B)
65	43	...	...	...	...	...	...	...	...	...	...	...	...	...	...	Eliminated
66	19	+	-	+	-	+	+	-	+	+	-	-	-	-	-	Parkinsonian (B)
67	5	+	-	+	+	-	+	-	+	+	+	-	-	+	-	Recovery incomplete (D)
68	20	+	-	+	+	-	+	-	-	+	-	-	-	-	-	Died
69	9	+	-	+	-	-	+	-	+	+	-	-	-	-	-	Recovery incomplete (C)
70	37	+	+	+	+	+	+	+	+	+	-	-	-	-	-	Died

no idea of how perplexing the diagnosis would in many instances prove to be, and, above all, he had no glimpse, whatever, of the appalling sequelae that were in a multitude of cases to follow in its train. Of all diseases it is most protean in its phenomena, greatly excelling hysteria in its power of simulation, and in its milder phases frequently being mistaken for it. So atypical has it become in symptomatology that no longer can the cardinal signs of fever, headache, diplopia and lethargy be depended upon in reaching an authentic diagnosis. Amongst the expressions of its many-sidedness in the primary attack, the most pronounced are fever, insomnia, delirium, maniacal symptoms, lethargy, abnormal muscular movements, myoclonus, choreiform movements, convulsions, vertigo, headache, neuralgic pains, ocular and facial palsies, diplopia, ptosis, nystagmus, disorders of respiratory mechanism, apoplectiform attacks, skin eruptions, early Parkinsonism or katatonia. As to the later manifestations, the syndromes are legion, and all kinds can be established, depending on localisation of the process in the central nervous system. Among the many complications, which appear at varying times after the initial attack, are disorders of sleep, Parkinsonism, perversion of conduct, psychotic and nervous disturbances, respiratory and circulatory derangements, ocular disorders, and endocrinal symptoms.

#### RE-GROUPING OF CASES.

The enquiry was begun with a provisional acceptance of the diagnosis of encephalitis lethargica in the whole series, as reported to the Medical Officer of Health. The observations have suggested a revision of diagnosis in some instances, and the conclusions have reference only to such cases as may now reasonably be regarded as belonging to this particular category of disease.

The course of the illness, as exemplified in the various sequelae, is epitomised in the following tables, where the series is grouped according to the more prominent manifestation of disorder. In Table II the grouping represents the state of the patients in March 1924; this is one year after the initial attack. In Table III the grouping represents their state in March 1928; this is five years later.

An attempt is made to describe the course of the disease, by transferring the cases from one group to another, according as the state has been found to have undergone change. Thus the groups represent (I) Recovery complete; (II) Recovery incomplete; (III) Perversion of conduct; (IV) Parkinsonians; (V) Died.

The method of re-grouping may be indicated by reference to the cases under the heading of "Recovery complete." In 1924 there were eight such cases in this group. In 1928 it was found necessary to transfer three of these to other groups, whereas examination of the rest of the series suggest the transfer of the other cases, who had not recovered at 1924, into the recovered group of 1928.

The results at the end of the first and fifth years' observations have been

Table II. *A constructive review of the condition of the patients in the spring of 1924.*

Italic numeral = Case number. Numbers in parentheses = Age at 1924.

GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
Recovery complete	Recovery incomplete	Perversion of conduct	Parkinsonians	Died
4. (29)	<i>Class A. Mental retardation</i>	1. (9)	<i>Class A.</i>	2. (11)
7. (48)	36. (9)	17. (8)	<i>Normal mentality</i>	3. (11)
14. (28)		52. (14)		6. (33)
28. (13)	<i>Class B. Mental instability</i>		8. (11)	21. (56)
32. (20)			20. (14)	27. (8)
42. (3)	9. (8) 59. (12)		24. (24)	34. (27)
43. (51)	16. (8) 64. (6)		25. (12)	39. (53)
62. (24)	19. (10)		37. (14)	40. (13)
			44. (23)	45. (20)
			66. (20)	47. (4)
	<i>Class C. Nervous instability</i>		<i>Class B.</i>	50. (43)
	10. (20) 33. (15)		<i>Abnormal mentality</i>	51. (7)
	11. (25) 38. (13½)			53. (5)
	13. (27) 41. (16)		15. (12)	55. (19)
	18. (20) 46. (10)		54. (11)	57. (19)
	22. (17) 49. (27)			63. (48)
	23. (24) 56. (31)			68. (20)
	26. (2½) 60. (6)			70. (37)
	29. (13) 61. (49)			
	30. (16) 69. (10)			
	31. (41)			
	<i>Class D. Physical defect</i>			
	(Not Parkinsonism)			
	12. (13) 67. (6)			
	<i>Class E. Physical defect +</i>			
	<i>Mental instability</i>			
	Nil			

Table III. *A reconstructive review of the condition of the patients in the spring of 1928.*

Italic numeral = Case number. Numbers in parenthesis = Age at 1928.

GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
Recovery complete	Recovery incomplete	Perversion of conduct	Parkinsonians	Died
7. (52)	<i>Class A. Mental retardation</i>	1. (13)	<i>Class A. Normal mentality</i>	17. (12)
28. (17)	26. (6½) 36. (13)	19. (14)	4. (33) 31. (45)	37. (17)
32. (24)			8. (15) *37. (17)	64. (9)
41. (20)	<i>Class B. Mental instability</i>		11. (29) 38. (17)	
42. (7)	9. (12) 60. (10)		24. (28) 43. (55)	
56. (35)			25. (16) 44. (27)	
62. (28)	<i>Class C. Nervous instability</i>		<i>Class B. Abnormal mentality</i>	
	10. (24) 30. (20)		16. (12) 54. (15)	
	13. (31) 33. (19)		*17. (12) 59. (16)	
	14. (32) 46. (14)		20. (18) *64. (9)	
	18. (24) 49. (31)		52. (18) 66. (24)	
	22. (21) 61. (53)			
	23. (28) 69. (14)			
	29. (17)			
	<i>Class D. Physical Defect</i>			
	(Not Parkinsonism)			
	67. (10)			
	<i>Class E. Physical defect +</i>			
	<i>Mental instability</i>			
	12. (17) 15. (16)			

\* Died

divided, as above mentioned, into five groups, and in each review the grouping corresponds.

*Group I. Recovery complete.* The patients in this group are those, who have apparently regained their normal health and have resumed their occupation, showing so far no residual signs of their previous illness.

*Group II. Recovery incomplete.* In this group are those, who have been left with some defect, but where the complication does not take the form of the more pronounced and characteristic states of Perversion of conduct and Parkinsonism following the disease.

*Group III. Perversion of conduct.* This group is composed of children, who have become abnormal in conduct and have undergone a complete change of character, showing signs of moral imbecility.

*Group IV. Parkinsonians.* In this group are those, who have, as a complication, developed Parkinsonism during or after the initial attack.

*Group V. Died.* This group comprises the cases which have proved fatal up to 1928.

#### GROUP I. RECOVERY COMPLETE.

It is impossible to state a definite period of time, when freedom from the danger of epidemic encephalitis can be regarded as permanent. Recovery, or apparent recovery, from the acute attack does not ensure that the patient will be wholly free from further trouble, and in this series it is found that one patient, after an apparent recovery of  $4\frac{1}{2}$  years, developed Parkinsonian symptoms. There is, however, no evidence of a relapse, nor recrudescence, nor extension of an infective process in the nervous system; but, rather, there is evidence of the appearance of new phenomena, which may present themselves months, or even years, after the acute symptoms of the initial attack have disappeared, and which would seem to be due to a breakdown of a mechanism, damaged in the acute stage of the disease. The presumption is that a complicated nervous system, although injured by disease, may survive the effects of impairment for a considerable time without showing disorder of function, and subsequently break down under strain. Such an assumption is in perfect accord with what is known to occur in heart disease.

In considering the recovered groups of 1924 and 1928 jointly, three types of cases are included:

(a) Those in which an uninterrupted and complete recovery has been made.

(b) Those of patients who have now recovered from complications, which were still present a year after the initial attack.

(c) Those of patients who, after an initial recovery, have developed complications after a year.

In the re-examination of the patients in 1928, it was found that, of the original eight recovered in the first review, there are only five who now remain



recovered (Nos. 7, 28, 32, 42, 62). Owing to the development of further symptoms after an initial recovery, it has been found necessary to transfer three patients, presumed to have been recovered in 1924, to other groups, two of these having developed Parkinsonism (Nos. 4, 43), and the third (No. 14) having become neurasthenic after pregnancy. There have been added to the recovered list, from the first review, two cases (Nos. 41, 56), both patients having recovered from complications which were still present a year after the initial illness, so that there are now altogether seven cases in this group.

Of those five patients, who have now made a complete and uninterrupted recovery, the first case (No. 7) is of a man of middle age, who showed symptoms at the onset of fever, drowsiness, diplopia, twitchings, and giddiness. His illness lasted for 6 weeks, during which time he was very nervous, although, while in hospital, he was suspected of exaggerating his symptoms. He is at present quite well, and has resumed his occupation of fireman on board an Atlantic liner.

The second case (No. 28) is that of a girl whose illness lasted for three weeks, and was characterised by drowsiness, pains all over the body, giddiness, vomiting, and diplopia, symptoms as of influenza. The girl made a rapid recovery, and was able to return to school shortly after leaving hospital.

The next case (No. 32) is that of a woman, whose illness was of 4 weeks' duration, with symptoms of drowsiness, lymphocytosis, increased pressure, and no diplopia. The patient was sufficiently recovered to return to service 2 months after her discharge from hospital. She married 6 months later, and has had one child. Owing to a contracted pelvis Caesarean section was performed at the birth. The child is healthy, and the mother made a good recovery. She remains well and fit for her household duties.

In the fourth case (No. 42) the initial illness was acute and subsided after eight weeks. The primary symptoms were lethargy, twitchings, lymphocytosis, increased pressure, marked Kernig's sign. Temp. 103° F. P. 130. R. 48. The lungs were clear to percussion, R. M. more harsh over left apex than right. The child made a gradual recovery, and was dismissed well from hospital. She has been at school since the age of 5, and is at present quite strong. Meningismus might be suspected in this case.

The fifth patient (No. 62) had mild symptoms in the initial stage, and altogether the illness lasted for 2 weeks. A few days before his admission to hospital, the patient had a fall from his cycle, and the illness commenced with drowsiness, diplopia, slight nystagmus, giddiness, and twitchings. In the initial stage he took a succession of fainting turns, after which he wept readily, and was confused in his ideas. After a few days of drowsiness he rapidly recovered, and later was dismissed from hospital well.

Of those two patients, who have now made a complete recovery from symptoms which were still present a year after the acute attack, the first (No. 41) had in the initial stage an illness lasting for 4 weeks, with symptoms of lethargy, headache, giddiness, lymphocytosis, but no diplopia. After his

discharge from hospital he had some weakness of the lower limbs and, for more than a year, salivation was excessive. This condition has now cleared up, and he is at present quite fit.

The other patient who recovered is a married woman (No. 56), in whom the onset was gradual, with drowsiness, headache, squint, giddiness, and vomiting. There was no fever and no diplopia, and the illness lasted for 6 weeks. She complained of giddiness and nystagmus for more than a year after leaving hospital, but later these symptoms disappeared and she is now well.

Of the three patients, who, after an apparent recovery, developed complications since the time of the first review, two have become Parkinsonians.

The first (No. 4), a police constable, developed Parkinsonism about  $4\frac{1}{2}$  years after the primary attack. His illness lasted for 4 months in the initial stage, and was characterised by pain in the right side of the neck, diplopia, nausea, giddiness, and lethargy, with intervals of delirium. He was able to resume his duties 6 weeks after leaving hospital, although for a few months he complained of pain in the right arm. He made a good recovery, and married 2 years after his illness; he has two children, both of whom are healthy. Some months ago tremors of the left arm were felt, and since then Parkinsonian symptoms have gradually developed. He has become stolid in appearance, speaks hurriedly in a thick monotonous voice, and there is excessive salivation. When walking he has a forward bend, and when sitting there is a tendency to droop from the waist. There is also a suspicion of stiffness in the left arm, and the eyesight is weak. His condition is such that it is doubtful if he will be able to continue his duties for any length of time.

The other patient (No. 43), who developed Parkinsonism, made an apparent recovery from her initial illness. She sustained a shock  $3\frac{1}{2}$  years after the onset, and, from that time forward, Parkinsonism has steadily progressed.

In the remaining case (No. 14) pregnancy was responsible for the development of nervous symptoms after an apparent recovery from the primary illness. This patient, a married woman, had an illness of 3 months' duration, with symptoms of delirium, restlessness, twitchings, and pyrexia. She made an apparent recovery, and at the end of the first year was seemingly well. A few months after the birth of a child she became emotional, irritable, and quick-tempered. She now suffers from violent headaches, accompanied by tremors and her eyesight has become weak.

After following out and investigating these cases of recovery it seems very doubtful, from the course of the illness and the subsequent recoveries, if all of the seven cases were of true encephalitis. Owing to the occurrence of these cases during an epidemic, and to the difficulty of diagnosis in the early stages of the disease, and also to the fact that, in the 1923 epidemic, medical practitioners were circularised regarding encephalitis, and were consequently on the *qui vive* for this "new disease," many may have been overhasty in the diagnosing and notifying of cases.

## GROUP II. RECOVERY INCOMPLETE.

This is the largest of the five groups, and has been sub-divided into five classes.

Class A. Mental retardation.

Class B. Mental instability.

Class C. Nervous instability.

Class D. Physical defect. (Not Parkinsonism.)

Class E. Physical defect + Mental instability.

*Class A. Mental retardation.*

This class is comprised of those cases showing signs of mental backwardness. In only two of the cases in this series is there mental retardation, and both of these are of children, this process having become evident in one child at the age of 9 years and in the other at the age of 6 years.

On re-examining this class in 1928 it is found that the original one case (No. 36) still remains from the first review; another case (No. 26) has now been added, having been transferred from the Nervous instability class, as it is evident, on interrogation, that the patient's mental processes are much slower than those of the average child of her years.

In the first case (No. 36) the child was acutely ill at the onset, being fevered, restless, delirious, and later lethargic. From the time of her illness a complete change of disposition was noted. From being bright and lively she became dull and apathetic, and, on her return to school 6 months later, she made little headway, and found great difficulty with her lessons. She lacked concentration and her memory was not good. There has been no improvement in the child's condition within the last 4 years. At present she is backward and dull, and shows signs of irritability and temper. Excessive salivation and respiratory disturbance (snoring) have been in evidence for about  $3\frac{1}{2}$  years.

The other case (No. 26) which has now been added was of a young child of 18 months at the time of her illness. She had initial symptoms of irritability, restlessness, wakefulness by night, and drowsiness by day. Nervous symptoms persisted for more than a year, but gradually wore off. She went to school at the age of 5, but made very slow progress. She still finds great difficulty in coping with her school work, and lately was held back a class.

*Class B. Mental instability.*

This class is composed of those cases, showing signs of abnormal mental excitability, without perversion of conduct or mental retardation. This milder and less distinct upset of conduct, involving a change in disposition, may be the only sign of incomplete recovery.

At present only one case (No. 9) out of the original five remains in this class. It has been necessary to transfer four cases to other groups, and to add one case from another class. Of the four cases which have been transferred,

three (Nos. 16, 64, 59) have become abnormal Parkinsonians, one patient died (No. 64), and the fourth (No. 19) has become perverted in conduct. The case (No. 60) which has been added showed nervous symptoms at the time of the first review, and was then in the Nervous instability class.

In investigating the series, it is found that this class comprises children, who are boys with one exception. In the first review the children varied in age from 6 to 12 years, and all uniformly showed, from the time of their illness, symptoms of marked restlessness with excitability, especially at night-time. In two cases (Nos. 16, 19), since transferred, the behaviour disorder assumed a more severe form, and, from the time of the acute attack, they showed extreme emotionalism, turning night into day, whistling and singing, tossing and burrowing in bed, tearing the bed-clothes, until they were utterly spent and became drowsy by day.

The following two cases illustrate the present condition of this class, showing the effect of encephalitis upon the development of the disposition of the child. Here the intelligence is good, there is no lack of moral ideas, but mental instability is evident.

In the first case (No. 9) the child had an illness of 3 months' duration with, in the initial stage, symptoms of insomnia, restlessness, twitchings, delirium, and strabismus. At the time of leaving hospital he suffered from fleeting apprehensions of emotional instability with great restlessness. Two months later he returned to school, but was nervous and fearful. He refused to leave the class-room at play-time, and informed his teacher he was afraid of someone going to do him harm. One day, when out walking with his mother, he suddenly ran ahead, always glancing backwards with a look of terror on his face; when at last she managed to get hold of him, he said, "There is someone chasing me, trying to do something to me." This abnormal condition lasted for over a year. Since his illness he has become extremely restless and emotional, showing marked eccentricities of conduct, and his disposition has changed completely. Prior to his illness he was shy and reticent, but now he will talk to anyone he meets; when in school he addresses his teacher in the middle of a lesson, and occasionally rises and walks round the class-room. In acquiring abstract knowledge he has made average progress. He is neither malicious nor vicious, but is affectionate and thoughtful in the home.

The second patient (No. 60) also shows peculiarities of conduct. His initial illness was characterised by delirium, restlessness, twitchings, and drowsiness, and lasted for 6 weeks. Following the illness a definite change of conduct was observed; from being shy and aloof, he became bold and obtrusive, and would brook no interference. He was quick-tempered, restless, and slept badly, and was unable to attend school for 8 months. Since his return to school his progress has been quite good, but the teacher complains he is difficult and quarrelsome, and at times forgets himself and where he is. On one occasion, during a lesson, he suddenly left his seat, walked to the window and exclaimed, "This is a fine day, Miss Smith." Under any emotional stress, such as a fit of

temper, salivation becomes excessive. His habits are good, and he is quite intelligent.

The following patient (No. 59), a boy, at present 16 years of age, now transferred to the Parkinsonian group—Abnormal mentality—is interesting on account of his having frequent lapses of memory. The boy showed a decided change of disposition from the time of his illness, becoming emotional, childish, and shunning his contemporaries. He repeatedly went for long walks, and afterwards had no recollection of his wanderings. On one occasion he took a walk without his boots.

*Class C. Nervous instability.*

This class embraces all those cases showing in a varying degree a residuum of nervous disturbances, including insomnia, drowsiness, irritability, tremor, headache, and weak eyesight.

Of the initial nineteen cases of the first review, twelve cases remain in this class (Nos. 10, 13, 18, 22, 23, 29, 30, 33, 46, 49, 61, 69). Seven cases have been transferred to other groups, and one case has now been added to the list, so that at the present time the class numbers thirteen. Of the seven cases transferred, in three cases the patients have become Parkinsonians (Nos. 11, 31, 38), two cases have made a complete recovery (Nos. 41, 56), one (No. 60) is now mentally unstable while another (No. 26) is mentally retarded. The case (No. 14), which has been added, is from the Recovered group, nervous symptoms having developed after pregnancy.

The majority in this class are adults, and it can be seen that complete recovery is the exception rather than the rule. Of those patients who have sufficiently recovered to allow them to return to work, all show residual signs of their previous illness, and complain of various forms of psychic and physical disturbances. After the acute attack of encephalitis has passed, it is common in this class to find a persisting insomnia, which in some cases lasted for months, and in a few cases for years, although, as a rule, the insomnia gradually disappeared within or shortly after the first year. Twelve patients in this class suffered from nocturnal insomnia after the acute stage, and, in all cases but two, this troublesome condition cleared up at times varying from 6 months to 2 years. In these two cases (Nos. 23, 31) insomnia still persists as a residuum.

Drowsiness is also found to occur as a late effect, but it is not a common sequel as is insomnia, having appeared only in four cases in this class, in two of which it is still evident (Nos. 18, 22), in the other two the patients have got rid of it within the first few months (Nos. 13, 30).

Of the nineteen cases in this class at the time of the first review, irritability occurred in twelve cases, weak eyesight in four cases, headache in three cases, and tremors were absent. In ten cases there was excessive salivation, and in one there was in addition respiratory disturbance (hyperpnoea). Of the thirteen cases at present in this class, irritability occurs in ten, weak eyesight occurs in eight, headache in five, and tremors in three. Eight patients have excessive

salivation, and two have also respiratory disturbance (clearing of throat and yawning).

From the above findings it can be seen that nervous symptoms, with the exception of insomnia, have increased during the five years.

In one case of this class (No. 61) it is interesting to note a sequela, simulating the acute attack 4 years after the initial illness. The patient was suddenly stricken down with severe headache, vomiting, and loss of power of the lower limbs, symptoms similar to those in the initial attack. For 10 days she remained in a lethargic condition, after which these symptoms gradually disappeared. At present she suffers from irritability, headache, and weak eyesight.

*Class D. Physical defect.*

This class is made up of those patients suffering from physical disabilities without signs of Parkinsonism.

There were originally two cases in this class, and of these two cases, one now remains (No. 67), while the other has been transferred to the Physical defect + Mental instability class. The latter case (No. 12), who, after her illness, became abnormally fat, has, since the first review, become mentally unstable in addition to this physical defect. She is extremely emotional, violent tempered, and shows unaccountable aversions to people, especially to men.

The remaining case (No. 67) is unique and of unusual interest, being the only case in the series with severe physical disability without Parkinsonism or mental disturbance. This case, of a girl aged 5, showed initial symptoms of fever, vomiting, headache, delirium, restlessness, drowsiness, twitching of the facial muscles, and strabismus. The child was very drowsy during the first few weeks of her illness, but was dismissed well from hospital 6 weeks after the primary attack. She remained at home for the next few months, and then resumed school. At that time, she complained of pain in the right leg, which gradually increased. Two years ago she was sent to a special school owing to her physical condition. The child tired very easily, and the mother then noticed a slight wasting of the right leg. This wasting became more pronounced, later the foot dropped and, consequently, walking became difficult.

At present the right leg is about half an inch shorter than the left, the muscles are atrophied, and the foot is dropped and turns inward. She flings her foot when walking. She complains a good deal of pain in the leg and stiffness and numbness on awakening in the morning; during the winter she suffers from chilblains on the soles of both feet. She is troubled with frontal headaches, and, when in bed, the head perspires freely. She sighs and yawns frequently; her eyesight is weak, and for the last 3 months she has worn spectacles. There is no excessive salivation, although this was slightly present after leaving hospital. She is quite intelligent, is well up to the average at school, and is equable in temper. On examination the knee jerks were elicited on both sides, the right somewhat brisk, and the temperature of the right foot was lower than that of the left; otherwise physical examination was negative.

*Class E. Physical defect + Mental instability.*

This class is composed of those suffering from physical disability, with in addition mental instability, but without signs of Parkinsonism.

At the time of the first review there were no cases in this class, but, at present, there are two, the first case (No. 12) having been transferred from the Physical defective class and the second case (No. 15) having been transferred from the Abnormal Parkinsonian class. The latter case is of considerable interest owing to its simulation of other conditions.

This patient, a girl, was 11 years of age at the time of the initial illness. The onset commenced with severe pain in the right arm and the right side of the back. The following day she complained of severe abdominal pain, which persisted for 7 days, and, during that time, the child was extremely constipated. She became fevered, restless, delirious, and drowsy, and was admitted to the surgical ward, diagnosed as a case of appendicitis. Fortunately, owing to the presence of myoclonic movements of the abdominal wall, she was saved from an unnecessary operation. The child remained in hospital for 6 months and, at the time of her dismissal, her speech was slurred, and there was ptosis of both eyelids present. A year later she suffered from nocturnal insomnia, and, owing to severe pain in the left leg, was unable to attend school. At this time Parkinsonism seemed to develop. There was a tendency, when sitting, to adopt a markedly crouching attitude and, when standing or walking, there was a decided stoop. The arms were held stiffly, and the eyes had a staring expression, winking seldom, with slight bilateral ptosis of the eyelids. The face was somewhat masked, and increased salivation was present. There was continuous twitching of both arms, the right arm especially being affected. Hyperpnoea was also evident, and had been present since the onset. Abdominal pain still persisted, and, regularly after meals, there was vomiting without nausea. After her illness, her teeth dropped out, one by one.

Within the last 3 years improvement has been gradual; abdominal pain and vomiting have disappeared, salivation has become normal, and twitchings of the arms have ceased, and, at the present time, there is no sign of Parkinsonism. In appearance she is ruddy and healthy, but is of stunted growth. The head and entire trunk are bent toward the left, with spasm of the spinal and occipital muscles. There is considerable lordosis and scoliosis, with a convexity to the right; the chest wall shows marked deformity (pigeon chest). Pain in the back of the legs has lately worn off. She becomes easily exhausted, and has not yet menstruated. A complete change of character has taken place; since her illness she has become mentally unstable, and is precocious, sly, impulsive, and quarrelsome. As in this case Parkinsonian symptoms, which appeared a year after the onset, subsequently disappeared, on retrospection it seems doubtful if this was a true Parkinsonian state.

## GROUP III. PERVERSION OF CONDUCT.

This group, though small, is not without interest, and is composed of children, in age ranging from 8 to 14 years, who show as sequelae personality changes and moral delinquency.

In the review of 1924 there were three cases in this group, but, at the time of the present review, only one of that number remains. Two patients (Nos. 17, 52) have developed Parkinsonism in addition to conduct perversion, and have been transferred to the Abnormal Parkinsonian class. One case (No. 19) has been added to this group, that of a boy who, previously being mentally unstable, has since become perverted in conduct.

The behaviour syndrome, as found in the post-encephalitic child free from severe neurologic symptoms, has some special features. In this group of children, encephalitis is followed by more or less severe conduct changes, although no such transformation has been observed in the adults of the series, in whom are mostly found physical conditions and psychotic changes. This change of personality and character in the child is due to the fact that the nervous system has been attacked during the formative period of development. The personality of the child is transformed by the virus of the disease, so that the ill-effects following such an infection are disastrous and far-reaching. In some cases, disorders of conduct have been such that these young people have been brought into conflict with the law, and ultimately consigned to mental institutions. These children are impulsive, destructive, and violent; they lie, steal, beg, swear, and show erotic and sexual tendencies. They are not feeble-minded in the technical sense, although mentally defective in respect of morals. Their intelligence is good, and this troublesome and dangerous behaviour would seem to be caused by an involvement of the emotional, rather than of the intellectual, tendencies; thus, after the commission of an impulsive act, regret is often immediately expressed (Nos. 17, 19).

Unless there are steps taken to provide proper supervision and training of these children, there is the danger that in the future crime may be rife amongst this community. On the other hand these young people, under right conditions, might be so trained that there would be a surprising reduction in undesirable behaviour.

The following case well illustrates the characteristics of this group. The patient (No. 19), a boy, 9 years of age at the time of his illness, had severe chorea of 7 days' duration in the initial attack. For over a year there was extreme restlessness and an inversion of the sleep rhythm with nocturnal excitement and drowsiness during the day. His character, from the time of his illness, changed completely, and, after the first year, he became unmanageable, and was expelled from school owing to bad behaviour. He is now completely beyond control, and it is most difficult to keep him indoors; if deterred in any way, he threatens to jump out of a three-storey window. He leaves home every day about 7 a.m., returning at a late hour, and spends his time



associating with men at the docks. He lies and steals, and, about 5 months ago, he was taken up by the police for stealing newspapers out of a shop and selling them in the street. After this he got a job with a milkman, but speedily was dismissed for appropriating the customers' money. He has violent bouts of temper, and cannot be thwarted in any way. He is constantly hitching up his stockings to the extent of tearing the tops off them. The boy is highly intelligent, his memory is excellent, and, in spite of his being absent from school for a considerable length of time, he is able to recall all he ever learned. He is bright and attractive in appearance; occasionally he becomes penitent, and seems sorry for his bad behaviour.

#### GROUP IV. PARKINSONIANS.

Parkinsonism is the most common and malignant form of residua, and can manifest itself at any time and at any age; once this state has been established, there is little hope of abatement, and remissions, if any, are fleeting. Most of the patients with this form of sequela realise the gravity of their malady, and feel themselves progressively growing worse. The young show no more recuperative power than those more advanced in age.

Parkinsonism has developed in eighteen cases of this series, and in eight of this number conduct changes are also present. Fifteen of these cases are below 30 years of age and three are above that age.

The Parkinsonian group is divided into two classes. Class A, Normal mentality; Class B, Abnormal mentality. The first class is composed mostly of adults, and the second class mostly of children, the latter having developed Parkinsonism in addition to Perversion of conduct.

##### *Class A. Parkinsonism: Normal mentality.*

The patients in this class show slight deviation from the normal, but they are not such as would, by common consent, be included amongst the mentally afflicted.

Of the seven patients originally in this class there are four still remaining (Nos. 8, 24, 25, 44). Three have now been transferred from this class, two of these having becoming Abnormal Parkinsonians (Nos. 20, 66), and the third (No. 37) having died. Five have been added, two (Nos. 4, 43), coming from the Recovered group, having lately developed Parkinsonism after an apparent recovery, and three (Nos. 11, 31, 38) having now been transferred from the Nervous instability class.

In many of these cases, the early phase of Parkinsonism was first recognised by a tendency to easy fatigue and a reduction of energy, with perhaps a slightly fixed facial expression. Further symptoms gradually developed with stiffness in the neck or in one or more limbs, drooping of the body, tremors of the arms or legs, difficulty and slowness in walking, rising, and sitting, monotony of speech, excessive salivation, and progressive fixity of the facial expression, until the typical Parkinsonian picture was presented. The con-

dition at this stage is sometimes stationary, but later steadily progresses; the rigidity increases, tremor becomes more marked, or is completely suppressed by the rigidity, saliva increases, and eventually the patient can no longer feed himself. At the end, emaciation is extreme, and, bed-ridden and helpless, he lies in a fixed position, staring into space.

All the patients display some disturbance in the psychic sphere, although the intellect is usually spared. To their physical disabilities is added a marked emotionalism, causing them to become a burden to themselves and to those around them. Some are hysterical, being readily exhilarated or depressed, alternately laughing and crying—from tears to laughter and from laughter to tears. Some are neurasthenic, being timorous, fearful, and tired, unable to create new thoughts or new ideas; they linger in bed most part of the day, and arise only to sit about listless and unoccupied. Others are worried, anxious, and harassed, showing an anxiety as to the ultimate issue of their trouble.

Among the group of Parkinsonians with normal mentality, only two (Nos. 4, 43) are at present able to carry on their usual occupation, although, probably before very long, these patients will require to discontinue owing to their increasing incapacity.

The following case of Parkinsonism, accompanied by neuralgias, is of special interest, being of rare occurrence. This patient (No. 44), a young woman, 22 years of age, had, in the initial phase, symptoms of drowsiness, delirium, vomiting, severe pain in the back of the head and neck, also pain in the feet and legs. She remained in hospital for 3 months, and during that time signs of Parkinsonism became evident; the face was mask-like and katatonia was present. After leaving hospital Parkinsonism gradually developed, although the neuralgic pains greatly lessened. She carried on a part-time job for about  $2\frac{1}{2}$  years, but was forced to discontinue owing to her physical condition. About a year ago there was a recurrence of severe pain in the back of the head and neck, shoulder blades, soles of feet, also in the joints of the large toes. This painful condition still persists, while Parkinsonism is now firmly established. In this case there is no past history of neuritis or rheumatism.

In another case (No. 11), that of a woman, 24 years of age, Parkinsonism manifested itself  $4\frac{1}{2}$  years after the initial attack, and, 6 months after the appearance of this syndrome, there was a sudden outburst of a maniacal nature. Immediately before the primary illness, the patient had a great deal of trouble, owing to the death of her fiancé, and to worry in business. At the beginning the illness was characterised by insomnia, delirium, headache, and diplopia. She gradually improved, and, after convalescing for a few months, was able to return to business. During the next few years she felt fairly well, except for a feeling of irritability and depression with occasional headache, until June 1927, when she complained of weakness and shakiness of the right arm and leg. Symptoms increased, and 3 months later Parkinsonism was pronounced and she was forced to give up work. In December 1927 there was a sudden attack of acute maniacal delirium, and she was confined to bed for

4 weeks. This acute condition passed off, but Parkinsonism increased, and at present she presents the typical picture of that syndrome.

*Class B. Parkinsonism: Abnormal mentality.*

The criterion of abnormal mentality in these patients is their mode of behaviour, including such aberration as suicidal impulse, necessitating treatment and supervision.

It is observed that all the cases in this class are of children with one exception, and, at the time of the first review, two patients (Nos. 15, 54) were in this class, one of whom still remains. It has been found necessary to transfer the other (No. 15) to the Physical defect + Mental instability class, as in this case symptoms of Parkinsonism have disappeared, while other physical disabilities have become very evident. Seven cases have now been added to the list, two cases (Nos. 20, 66) from the Normal Parkinsonian class, three (Nos. 16, 59, 64) from the Mental instability class, and two (Nos. 17, 52) from the Perversion of conduct group, so that altogether there is a total of eight cases in the class.

Although in the majority of the children showing Parkinsonism the same physical symptoms characterise the disease as in the adults with this form of sequela, there is, however, a difference in the mental attitude. Mental deterioration is found to take place prior to the development of the Parkinsonian syndrome, and, with the advancement of Parkinsonism, the psycho-motor excitement grows less, until, eventually, there is a cessation of impulsive acts. In all the children of this class, changes occur both in character and behaviour, even to the extent in some cases of utter perversion. Four of the children coming under this class (Nos. 16, 17, 52, 54) exhibit misbehaviour of the lowest order, with a history of explosive conduct, such as outbreaks of violent temper, cruelty, lying, thieving, profanity, destructiveness, and sexual abnormalities. Not all of these symptoms occur in one child, but this enumeration suggests the condition.

The following case (No. 17) is a typical example of Parkinsonism in a child, with mental deterioration. The illness commenced with fever, drowsiness, hallucinations, tremors, and strabismus. At the time of leaving hospital, 6 months after the onset, the child was still drowsy and very emotional and querulous. On returning to school, 2 months later, she was found to be uncontrollable and unresponsive to discipline, so much so that, after 3 months, it was necessary to have her removed. She was constantly in trouble, and her habits were of the lowest; she lied, stole, used obscene language, played about with faeces and urine, was violent and showed sexual abnormalities. At times, she seemed conscious of her sad behaviour, and on one occasion after striking a child, prayed, "Oh God, make me better." She was ravenous for food, and, although well fed at home, would enter neighbours' houses and seize anything in the way of food, to the extent of snatching it from people's mouths; at times her thirst was abnormal. She was certified for Woodilee Asylum 16 months

after the initial illness, and on two occasions she managed to escape. About this time Parkinsonism began to develop, and physical deterioration gradually progressed, whilst malbehaviour lessened, until finally there was a cessation of impulsive acts. Since December 1927 she has been confined to bed, a helpless wreck, in the grip of Parkinsonism and lethargy is pronounced. In this case the family history is not good, the child's father being alcoholic and the mother neuropathic.

The second case (No. 52) is that of a boy, whose misbehaviour includes a long list of misdemeanours and crimes. His illness commenced with headache, sleeplessness, later drowsiness, vomiting, diplopia, fever, twitchings and vertigo. After an illness of 5 weeks he was discharged well from hospital. He then returned to school, but his behaviour was so bad that he was expelled 2 months later. After the age of 14 he was employed by different tradespeople and was dismissed, time and again, owing to thieving, lying and violent temper. His appetite was ravenous, and he could rarely be satisfied. He was continually in trouble with the police on account of his interfering with young girls; on one occasion he almost strangled a shop-girl, but was deterred through timely intervention. Two years after his illness, he applied and was appointed to a Naval Training Ship at Devonport. He remained there for 5 months, but, as he was found to be homosexual and generally degraded in conduct, he was certified as mentally unsound. At this time a tendency to Parkinsonism was observed. When visited in Hartwood Asylum in the spring of 1928, he was going about and performing his quota of work daily. There was then evidence of the advancement of the Parkinsonian syndrome; the face was expressionless, the gaze fixed, speech difficult, but there was no loss of co-ordinated movements. He was reported to be emotional, irritable, childish and facile, with an underlying astuteness. He requires careful watching on account of homosexual practices. This patient is an illegitimate child, and his parentage is not good. He showed signs of abnormal conduct prior to his illness.

In considering the relation of the onset of Parkinsonism to mental degeneration, it is found that, in all the children of this class, mental degeneration preceded Parkinsonism, and in only one case, that of an adult, mental degeneration followed Parkinsonism. All seven children exhibited deterioration within the first few months after the initial illness, and, in the case of the adult, mental deterioration became evident about 7 months ago. This patient, a young woman (No. 66), has become very depressed owing to her physical condition and to her inability to use her hands. She is constantly wailing, "Oh my hands, my hands," with which she has become obsessed. In August 1927 she attempted to commit suicide, taking a razor to her throat, and at that time was confined for a short period in a mental institution.

From the study of the asylum cases, the family history has been found to be unfavourable to the patient. In one case (No. 20) the mother is a confirmed drunkard and shows maniacal tendencies; in another case (No. 17) the mother is neuropathic, having frequent periods of depression, and the father is

alcoholic, and was so before he married; in a third case (No. 52) there is also a family taint, the boy being illegitimate, and the father belonging to a low and degraded class. In still another case (No. 66) the family history shows a neuropathic strain in many members, one uncle being confined to Gartnavel Asylum for the past 3 years, while other relatives show signs of mental instability. Thus, it would seem that the underlying personality and the hereditary factors play an important part in this expression of the disease, and deterioration in children, traced to heredity, is greatly aggravated by the infection, although it is also produced to a considerable extent in cases with no bad history. Children below the age of 16 are more prone to severe conduct changes, becoming less vulnerable after that age.

An attempt has been made to establish the length of time between the initial attack and the onset of the Parkinsonian syndrome. Although it is often assumed that a Parkinsonian condition is a late manifestation of epidemic encephalitis, it is found, in the cases under review, that it can also occur immediately after the initial attack, or even during that time as a complication. In some cases, after the recession of the acute phase, it was observed that a definite normal interval intervened, which varied from a few months up to 5 years, while, in a few cases, the development of symptoms was so gradual and so insidious that it is difficult to fix an exact time of onset.

There are altogether eighteen cases showing Parkinsonism in the series, and the following observations give approximately the time of onset of the syndrome in relation to the acute attack.

Four patients developed Parkinsonism during or immediately after the initial attack (Nos. 8, 20, 37, 44).

Four patients developed Parkinsonism within the first year of the initial attack (Nos. 24, 25, 54, 66).

Two patients developed Parkinsonism within the second year (Nos. 17, 64).

One patient developed Parkinsonism within the third year (No. 52).

One patient developed Parkinsonism within the fourth year (No. 43).

Five patients developed Parkinsonism within the fifth year (Nos. 4, 11, 16, 38, 59).

One patient developed Parkinsonism within the sixth year (No. 31).

It is, therefore, evident that Parkinsonism is liable to occur at any time and at any age, this phenomenon having appeared in patients varying in age from 6 years (No. 64) to 53 years (No. 43).

#### GROUP V. DIED.

This group comprises the cases which have proved fatal up to 1928.

Apart from the eighteen cases, which ended fatally during the first few months of the illness, there are only three cases in which death has been delayed for much longer periods. Within the first year the earliest death occurred 6 days after the onset and the latest at 5 months. Of the eighteen deaths occurring within 5 months of the onset, in one case (No. 70) pneumonia

was contracted 7 days before the end. Eleven of the patients were over the age of 16 years, and seven were under that age. Of the three deaths, which occurred after the first year, Parkinsonism was found to be the terminating factor. Two of the patients were under the age of 14, and the other was 18 years of age.

Parkinsonism in the first case (No. 37) developed during the initial attack, and lasted for 4 years and 3 months; in the second case (No. 64) this sequela developed shortly after the first year, and lasted for 2 years and 9 months; and in the third case (No. 17) the sequela developed shortly after the first year and lasted for 4 years.

#### ELIMINATED CASES.

In reviewing the whole series, it has been considered necessary to eliminate five cases, as, after these cases have been traced and followed out, there does not appear sufficient ground for including them in the series.

The first case (No. 35) is that of a man, aged 81, whose initial illness was characterised by drowsiness and hallucinations, the patient imagining he was very poor, and that he had no money for food. He was full of fears, and believed he was being pursued by enemies. There was no defect of speech, nor any signs of paralysis. Towards the end he was in a state of coma, and died 3 months after the onset. In considering the course of the disease and the age of this patient, it would seem that this was a case of arterial brain disease, and a post-mortem examination would have been required before a diagnosis of lethargic encephalitis could be regarded as final.

The second case (No. 65) is that of a single man, aged 43. This patient had held a position in West Africa for 24 years previous to his illness. In 1918 he had an apoplectiform seizure, and was invalided home; since then he has done no work. His illness, in 1923, commenced suddenly with symptoms of headache, vomiting, coma, and right hemiplegia; later there was mental confusion with some difficulty of speech and convergent strabismus. After 5 weeks in hospital he made gradual improvement. At present he leads a fairly active life, reading, walking, and golfing. His memory is very poor, and he is unsocial, eschewing company; this he states is due to the fact that, while in conversation, he frequently forgets what he wishes to say. On examination it is found that the pupils are equal, and react well to light and on accommodation. Bilateral nystagmus is present, but more pronounced on the right side. The knee jerks are elicited on both sides, right somewhat exaggerated. There is no Babinski sign nor ankle clonus. There is slight tongue tremor, and also tremor of the right hand on extension. It seems very doubtful whether this is a case of encephalitis lethargica, but it would require careful observation of the development and course of the illness to determine a positive diagnosis.

The third case (No. 5) is of a man, 24 years of age. Restlessness, confusion of mind, and incoherence of speech characterised the onset. The patient was 5 weeks in hospital, and later was transferred to the asylum. He had served in the war, and had suffered from shell-shock. After the initial phase of the

illness, he became childish, incoherent, and completely disorientated. His habits were dirty and there was a marked degree of dementia. He had seizures of the general paretic character, showed a considerable amount of paresis, and exhibited many of the physical signs of general paralysis of the insane. Wassermann reaction of the blood and C.S.F. were positive. The patient died a year after the initial attack, having been confined to the asylum for 11 months. This is evidently not a case of encephalitis lethargica, and, from the symptoms expressed, a diagnosis of general paralysis would seem justifiable.

The fourth case (No. 58) is of a man, aged 26, whose illness, in the initial stage, showed symptoms of headache, dimness of vision, general pains, and weakness. After leaving hospital, the patient was at home for several weeks; during that time he developed convergent strabismus, and complained of weakness of the left arm and leg. He returned to hospital for nine months, and, according to the statement of his wife, he was treated at that time by "injections into the arm." From the beginning of his illness there was a gradual loss of power, both arms and legs ultimately becoming paralysed. Towards the end he lost the power of speech and became totally blind. He died 18 months after the onset of his illness. In following the general trend of this case with its ultimate blindness, syphilis would seem a more likely diagnosis than that of encephalitis.

The last case (No. 48) is that of a man, 37 years of age. The initial symptoms were fever, giddiness, drowsiness, and strabismus of the left eye; the speech was slow and slightly slurred, and the facial expression mask-like. The patient was in hospital for a month, during which time he became steadily worse. Twitching of the arms was present, and katalepsy was easily produced. On examination, the chest was clear to percussion, R.M. vesicular, and no increased V.R. or V.F. Reflexes were equal on both sides, but slightly exaggerated. A fortnight before the end, the patient suddenly had a fit. He died 5 weeks after the onset. A post-mortem examination was held, and the findings showed a tumour of the lung with metastases in the brain and in the right suprarenal.

#### POLIOMYELITIS—INFLUENZAL ENCEPHALITIS—LETHARGIC ENCEPHALITIS.

##### A COMPARISON AND A CONTRAST.

Fever, headache, vomiting, squint, convulsions, and delirium may constitute, individually or severally, the signal of acute infection, not only of the brain or its membranes, but of such diverse organs as bone, lungs, or kidneys; they may also herald the onset of the more general toxæmias of measles, scarlet fever, or typhus. Acute cerebral signs and symptoms do not, by any means, justify a diagnosis of cerebral disease. It is, as a rule, in the course of the disease, when the confusion of the incipient attack has abated, that the phenomena, which point to the nature of the infection, begin to reveal themselves.

It is no wonder, then, that, when lethargic encephalitis appeared, the equivocal character of its initial expression should have suggested a relationship with poliomyelitis and cerebral influenza. Poliomyelitis was an endemic form of acute nervous disease, which sometimes became epidemic; it was a disease with which the public and physicians were familiar; moreover, when it occurred epidemically, the cerebral variety was by no means rare. On the other hand, cerebral influenza was a well-recognised expression of that infection, and its incidence at a time when the disease had become pandemic was not unexpected.

In these circumstances it is not surprising that attempts should have been made to correlate the clinical phenomena of the "new disease" with those of other infections with which doctors were already acquainted, and to recognise in it an aberrant variety of the more familiar infections.

It soon became apparent, however, that the new disease had a way of its own. Despite its resemblance to other cerebral disorders, it followed a course which, however varied, presented features of distinction, which rendered its recognition a matter of comparative certainty. These features were not necessarily the lethargy or torpor, not in themselves peculiar to the disease; nor yet the chorea and myoclonus, which, in their atypical characters and epidemic incidence, constituted a strange and novel phenomenon; it was rather in the aftermath or relic of convalescence that the derangement revealed its truly original character. The records of medicine contain no reference to acute cerebral disease, followed by such characteristic sequelae, as have been noted and described in the "perversion of conduct" in children and in the "Parkinsonian syndrome" in children and in adults.

There is no difficulty at this time of day, despite the fragmentary and inadequate character of our knowledge of the relation of function to the structure of the nervous system, in recognising the broad outlines which differentiate lethargic encephalitis, poliomyelitis and influenzal encephalitis from each other; for although, in fulminating forms of each infection, the anatomical degeneration may be diffuse, in average cases the pathological changes are localised more or less to regions characteristic for each infection. Thus, in acute poliomyelitis, inflammation is mostly confined to the anterior cornual cells and involves, as a consequence, the disappearance of the somatic motor fibres to voluntary muscle. In the encephalitis of influenza, the mischief predominates in the superficial grey matter of the cerebral cortex, while, in lethargic encephalitis, the foci of primary reaction lie, as a rule, in the mid-brain, in the hypothalamus, and in the basal nuclei of the cerebrum.

Elementary though such an anatomical conception may appear to be, it provides a key to the characteristic course pursued by each disease, as it emerges from the confusion of the initial derangement. Once the inflammatory and destructive phase has ceased, the organism tends to rehabilitate itself in response to the activity of those nervous structures that have not been destroyed.



In the case of acute poliomyelitis, the pathological conditions are simple, and are easily correlated with the clinical effects. The loss of anterior motor cells and fibres involves a very simple organic defect. These cells and these fibres are peripheral, and their disappearance does not prejudice the efficiency of any of the complicated internal mechanisms of neural integration. The defects, which follow the disease, are due exclusively to the paralysis and atrophy of voluntary muscle, and, as the extent of the physiological defect is proportionate to the amount of anterior cornual lesion destroyed, it is not difficult to formulate a prognosis, shortly after the acute illness has passed off.

With the involvement of the cerebral cortex in influenzal encephalitis, it is not surprising to find a clinical picture in which are portrayed defects and instability of psychic functions. The prolonged convalescence, characterised by loss of memory, asthenia and emotional depression, extending over weeks, months and even years, is the hall-mark of a disease, which, more than any other, carries in its train those signs and symptoms which commonly come under the designation of toxic neurasthenia. Whereas, in poliomyelitis, recovery consists in the restitution of somatic movements that have been paralysed through muscular wasting, convalescence from influenzal encephalitis is a process less defined, and consists in the gradual rehabilitation of mental and constitutional strength. While, in the former, the defect is essentially motor and to be met by mechanical therapy, in the latter, the anomalies are mainly visceral and psychic, and subject to therapeutic measures of a more general character. In each case, however, there is a tendency from the commencement, if not to recovery, at least to gradual improvement.

In striking contrast with these is the encephalitis of the lethargic type; for, however closely it may resemble them in the initial stages, and this is not always so, its later course and the relics of its primary damage claim for it a special place in the taxonomy of disease. The kaleidoscopic character of the clinical picture, as revealed in the foregoing review of the 1923 epidemic, finds no parallel in any other disease, not even in the protean expression of syphilis. The nature of the onset is no index as to what may follow. What appears to be a satisfactory convalescence, may only be a quiescent phase preceding the onset of one of the several sequelae, which terminate in permanent disability. Physical disability, change of character and disorders of nutrition, emerge in a manner which is bewildering in our present knowledge of the structure and function of the nervous system.

There are, however, biological considerations which suggest an organic basis for the peculiar features that distinguish the disease and its sequelae. Poliomyelitis, it has been noted, affects the peripheral motor nerves to voluntary muscle, and involves a comparatively simple interference with organic unity. The virus of influenza, when it selects the central nervous system, attacks the cerebral cortex and is responsible for psycho-neurotic disorders, related to derangement of the "higher centres" of neural integration. Lethargic encephalitis, while it may be diffuse like the other two forms, has a decided

preference for the mid-brain, the hypothalamus and the basal nuclei of the cerebrum. Although the anatomical and physiological relations of the constituent elements of these regions are still obscure, it is well known to biologists that they are the oldest and most fundamental parts of the nervous system. They contain the main centres of integration for the nervous regulation of both the visceral and somatic functions of the organism. The hypothalamus is the site of closely allied connections of the nervous mechanisms that control the organic systems of digestion, circulation, and respiration. Subsidiary centres of respiration and of vasomotor activity have been localised in the medulla, but there is experimental evidence to suggest that, in the hypothalamus, all the systems, which have to do with nutrition, are connected in a complicated integration. On the other hand, somatic movements, as contrasted with visceral reactions, are regulated and co-ordinated in their automatic activity by a compendium of reflexes, whose nuclear centres lie in the mid-brain and in the base of the cerebrum. These reflexes comprise the controlling and executive mechanism, which mediates between the higher associational centres of the cerebrum and the lower effector mechanisms of the brain-stem and spinal cord, to which the anterior cornual cells are related. On their afferent side they are related to the eyes and to the vestibular apparatus, and are thus concerned in the adjustment of conjugate deviations of vision, in the maintenance of equilibrium and in the automatic control of posture and of rotatory movements of the head and limbs in co-ordinate relation to movements of the axial skeleton.

When it is remembered that the main lesions of lethargic encephalitis are situated in the mid-brain, basal nuclei and hypothalamus, just in those centres concerned in the preservation of organic harmony as a whole, it is not difficult to appreciate, in a general way, the variety and extent of complications, which this devastating malady carries in its train. It selects just that part of the nervous system whose functional derangement might seek an expression in the greatest possible varieties of directions, and it is just this conception of a functional derangement which affords a clue to a rational understanding of what must otherwise appear to be a chaotic series of sequelae.

This conception of a functional nervous derangement, associated with organic destruction of tissue, is well established in British neurology, and dates back to Hughlings Jackson. It implies, in the present context, that the various sequelae, so far from being related each to the destruction of a particular locus, are the individual expressions of the disorder of a complicated central mechanism, injured and partially destroyed in the initial inflammatory process, and dislocated subsequently by the exhaustion of stress or strain or shock.

In this way, alone, would it appear to be possible to reduce to a common biological basis such heterogenous disorders as excessive salivation, profuse sweatings and respiratory tics on the one hand, and tremors, somatic tics, giddiness and all the phenomena of Parkinsonism on the other. In this way,

also, it is possible to understand that a disease, which commences with diplopia, squint, chorea, spasm, and myoclonus, may develop into a disability, characterised by defective posture and disorders of automatic movement.

Bearing these considerations in mind, it is not surprising that emotional instability should be a feature of the disease. Those reactions, known to the psychologist as emotional, and associated with abnormal excursions of excitement and depression, are supposed by some to be the expression of exaggerated or diminished activity of the central regulating mechanisms at the base of the brain. It is, in any case, a well-recognised fact that abnormality of movement, somatic and visceral, is the fundamental and palpable evidence of emotional disorder for which, indeed, the term "emotion" is literally a verbal symbol.

Retardation, impulse and automatism are characteristics of emotional disorder, affected, as it may be, by varying states of abnormal feeling. The retardation of the victim of Parkinsonism, whose intellect is usually unimpaired, is a perfect example of psycho-motor impediment. The impulse of the naughty child exhibits the licence of excitement, in which the will no longer represents the control of rational intelligence or of disciplinary habit. Criminal offences suggest the automatism of epilepsy and of hysteria, while salivatory exaggeration and respiratory tics represent corresponding excesses in the vegetative system.

It is highly probable that uncontrollable perversion of conduct, confined as it is almost exclusively to children, is to be related to disorder of basal centres. Attacked during the formative period of development, there is an interference with those constructive processes, whereby the instinctive or phylogenetic reactions of the most fundamental parts of the brain come gradually under the influence of the higher cortical centres, concerned with the evolution of rational intelligence. It is a matter of great significance that, whatever may have been the case in epidemics elsewhere or in other epidemics in Glasgow, mental defect, in the abstract sense of intellectual deterioration, was practically non-existent in the epidemic under review. It is feasible to suggest that the difference between children and adults, in respect of aberrant behaviour, is to be construed in the light of the difference in the stage of nervous and mental development at which the disease has occurred. In the case of children, dissolution of neural integration of basal centres has been produced before the higher faculties have assumed control; in adults, behaviour has already become regulated by precept and habit, so that the licence of instinctive reaction remains under the control of social custom.

The relatively greater frequency in young people of sequelae, involving the endocrine system, is also to be correlated with the incidence of the initial disease in the formative period of life; and these sequelae have been observed in eight of the cases under review, all of whom were children at the time of the onset. Of the varied forms of endocrine disturbances, obesity, menstrual irregularities, amenorrhoea, abnormal appetite, polydipsia, and polyuria are all found to occur.

Obesity, as a residual, is present only in one case (No. 12), that of a girl, aged 12. The initial illness was characterised by lethargy, and this condition still persists. A year after the illness it was noted that there was a gradual increase in subcutaneous fat, although, previous to the illness, the patient was inclined to be thin. In 1924, at the age of 13, her weight was 44.9 kg. and her height was 135 cm. An operation for appendicitis was performed 4 years later, and at that time several layers of fat were removed from the abdomen. In 1928, at the age of 17, her weight was 73.6 kg. and her height was 150.4 cm. Her appetite has been normal throughout.

Menstrual disturbances, it has also been noted, have occurred in girls when they reach the critical age at which normal menstruation should take place, and this abnormality is present in three cases of the series. An example of Parkinsonism in association with this disorder is illustrated by the following case (No. 8), that of a girl, at present 15½ years of age, whose illness commenced 5 years ago with sleeplessness, excitability, and twitchings. Parkinsonism developed immediately after the acute phase, and has steadily progressed, until now, when a state of helplessness has been reached. At the age of 14, menstruation took place on two occasions, but since then has not occurred. In the case (No. 12), previously described, that of a girl at present aged 17, there have been only two periods of menstruation, which happened at the age of 16. The third case (No. 15) is that of a girl, who is now 16 years of age, and has failed to menstruate.

In the case of five children (Nos. 1, 17, 52, 54, 59), diabetes insipidus is very marked and all of these children have voracious appetites, rarely being satisfied; two patients (Nos. 1, 17) have in addition abnormal thirst, accompanied by frequency of micturition. One of the most pronounced cases, showing this sequela, is that of a boy (No. 1), now aged 13, who, after his illness, developed an abnormal craving for food after having full meals at home. He goes begging round doors and, during the night, arises and devours all he can lay hands on; he drinks an excessive amount of water and has frequency of micturition.

The assumption that functional instability of the complicated basal mechanism, induced by partial dissolution of neural integration, explains in a superficial way the sequelae of encephalitis, finds support in the occurrence of such sequelae after knocks, shocks, and blows. This is clearly demonstrated in one case of this series (No. 43), a married woman of middle age, who, having made an apparent recovery from the initial illness, was able to resume household duties for 3½ years, when, suddenly, she sustained a shock, news being broken to her during the night of the serious illness of her daughter. Immediately after this happening symptoms of Parkinsonism appeared, and since have steadily developed.

[There is satisfactory evidence to show that in six cases of the series (Nos. 1, 9, 11, 17, 66, 69) a knock, shock, or blow has also acted as a precipitating factor in bringing about the onset of encephalitis, as in all six cases the

initial symptoms appeared almost immediately after the occurrence of some form of trauma. A history of a fall has been given in four other cases (Nos. 24, 43, 54, 62) and it would seem that here the fall was the direct outcome of a flaring up of the infection, as, shortly after the accident, acute symptoms manifested themselves.]

In the foregoing review an attempt has been made to portray in broad outline the main features of the Glasgow epidemic of encephalitis of 1923. The review is in no sense an intimate neurological study. It is sought, rather, to furnish a general impression of the clinical phenomena, as they first presented themselves, and to follow the picture, as it unfolded itself in the succeeding phases, one year and five years later. In this way there has been thrown into relief the characteristics which distinguish it from poliomyelitis and the encephalitis of influenza.

A critical summary of the sequelae reveals and illustrates the hypothesis that they are to be explained, not on the assumption of a progressive lesion of the nervous system such as general paralysis, but on the assumption of an initial and permanent damage to the complicated neural mechanism of the mid-brain, hypothalamus and basal nuclei. The consequent instability of the mechanism renders it prone to give expression to various forms of disability, depending on ill-defined proclivities, one of which is associated with the age of the patient at the time of the initial attack. However inscrutable its origin, however incalculable its course or obscure the conglomeration of its individual manifestations, there is no difficulty in recognising the scar it leaves on the health of the community.

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# THE SPERMICIDAL POWERS OF CHEMICAL CONTRACEPTIVES.

## II. PURE SUBSTANCES.

By JOHN R. BAKER, M.A., D.PHIL. (OXON.).

(With 3 Text-figures.)

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### INTRODUCTION.

THIS is the second of a series of papers on an extensive investigation of chemical contraceptives, undertaken at the suggestion of the Birth Control Investigation Committee and financed by it. I wish to thank the Committee for its continued support, and in particular Dr C. P. Blacker and Prof. J. S. Huxley, who have helped me in many ways. Prof. E. S. Goodrich, F.R.S., has kindly allowed the whole of the work to be done in the Department of Zoology and Comparative Anatomy at Oxford. That the work has resulted in the discovery of some very spermicidal substances must be attributed largely to the fertile suggestion of Prof. J. B. S. Haldane that I should investigate substances which reduce surface tension. Dr H. M. Carleton has always been generous with valuable advice. Dr F. H. A. Marshall, F.R.S., has been kind enough to read the proofs. The makers of the various commercial pessaries have most kindly given me quantitative analyses of them, but do not allow me to publish them. The qualitative analyses are not secret. I wish to thank the manufacturers for their co-operation.

The first paper in this series (Baker, 1930) dealt with the effects of commercial pessaries on guinea-pig sperms. An investigation of their effects on human sperms is almost complete, and will form the third paper in this series. The present paper is concerned not with commercial pessaries, but with chemically pure substances, free from any vehicle, diluent or foam-producing

mixture. It is only by an investigation of this sort that one can properly interpret the effects of commercial pessaries, and find new substances for commercial use. The investigation was conducted with guinea-pig sperms, on account of the impossibility of procuring sufficient human semen for this investigation as well as for the investigation of pessaries. On the whole guinea-pig sperms seem to react to spermicides in much the same way as human ones. I hope in the future to undertake an investigation of this subject. A coefficient might be found, whereby one could calculate directly at what concentration a given substance would kill human sperms, if the concentration at which it kills guinea-pig sperms were known. This would be a very great convenience. The guinea-pig was selected because it is easily kept and bred in the laboratory, matures quickly, remains fecund throughout the year, has a conveniently large epididymis, and produces very active sperms.

It appears that chemical contraceptives are coming to be regarded as unreliable, and only suitable for use in conjunction with other methods. That this is so must be due largely to the lack of physiological investigation of the subject. There are two ways of studying chemical contraceptives. Firstly there is the clinical method of asking women for their experience with various pessaries and tabulating the results. This has been done on a large scale by Dr Marie Stopes and others, and no one will deny the importance of their work. The objection to this type of investigation is that it is of necessity an experiment without controls. The second method of studying chemical contraceptives is the physiological one which I have adopted. The objection to it is that the experiments are performed in glass tubes and not in the vagina. Whilst admitting that this objection has weight, I must protest against the idea, quite unfounded on observation, that the vagina is a sort of chemical laboratory, equipped to change the chemical constitution of every substance placed within it. The great advantage of the physiological method is that every experiment has a control. Further, new substances may be tested without unwarrantable risks on women. The truth surely is that both the clinical and the physiological method should be used, and workers in neither field should disparage the research of those in the other.

Throughout this paper sperms will be spoken of as "killed" if they are completely immobilised by any substance at the temperature of the body. This is done because it has not been shown that any substance, with the possible exception of hydrogen ions, can immobilise Mammalian sperms at the temperature of the body without preventing them from becoming capable later of achieving fertilisation. The words "spermicide" and "spermicidal" are preferred, on grounds of euphony, to "spermatocide" and "spermatocidal."

#### THE SUBSTANCES TESTED.

Substances already used as chemical contraceptives, substances thought likely to be useful, and certain very poisonous substances were tested. The latter were tried for purposes of comparison only. Many friends were kind

enough to suggest substances for trial. Prof. Haldane's suggestions are discussed below. Thirty-six substances were investigated in such a manner that it is possible to place them all in the order of their spermicidal powers. They are arranged alphabetically below. So that there may be no doubt whatever about what substances are meant, the chemical formula of each is given.

Acetic acid	$\text{CH}_3\text{COOH}$
Ammonium chloride	$\text{NH}_4\text{Cl}$
Boric acid	$\text{H}_3\text{BO}_3$
Citric acid	$\text{C}_6\text{H}_4\text{OH}(\text{COOH})_3 \cdot \text{H}_2\text{O}$
Cresol	$\text{C}_6\text{H}_4 \cdot \text{OH} \cdot \text{CH}_3$
Dioxyquinolin sulphate	$(\text{C}_8\text{H}_6\text{NOH})_2 \cdot \text{H}_2\text{SO}_4$
Ethyl alcohol	$\text{C}_2\text{H}_5\text{OH}$
Formaldehyde	$\text{HCHO}$
Hexamine	$(\text{CH}_2)_6\text{N}_4$
Hexyl resorcin (Caprokol)	$\text{C}_6\text{H}_5(\text{OH})_2(\text{CH}_2)_5\text{CH}_3$
Hydrocyanic acid	$\text{HCN}$
Iodine	$\text{I}$
Lactic acid	$\text{CH}_3\text{CHOH} \cdot \text{COOH}$
Magnesium sulphate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
Mercuric chloride	$\text{HgCl}_2$
Phenol	$\text{C}_6\text{H}_5\text{OH}$
Potassium aluminium sulphate (anhydrous)	$\text{K}_2\text{SO}_4 \cdot \text{Al}_2(\text{SO}_4)_3$
Potassium borotartrate	$\text{KBO} \cdot \text{C}_4\text{H}_4\text{O}_6$
Potassium butyrate	$\text{KC}_4\text{H}_7\text{O}_2$
Potassium capronate	$\text{KC}_6\text{H}_{11}\text{O}_2$
Potassium cyanide	$\text{KCN}$
Potassium oxyquinolin sulphate (Chinosol)	$\text{K}_2\text{SO}_4 \cdot (\text{C}_8\text{H}_6\text{NOH})_2 \cdot \text{H}_2\text{SO}_4$
Potassium permanganate	$\text{K}_2\text{Mn}_2\text{O}_8$
Quinine bisulphate	$\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2 \cdot \text{H}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$
Quinine hydrochloride	$\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2 \cdot \text{HCl} \cdot 2\text{H}_2\text{O}$
Quinine urea hydrochloride	$\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2 \cdot \text{HCl} \cdot \text{CO}(\text{NH}_2)_2 \cdot \text{HCl} \cdot 5\text{H}_2\text{O}$
Saponino	$\text{C}_{18}\text{H}_{27-28}\text{O}_{10}$
Sodium citrate	$2\text{C}_6\text{H}_5\text{OH}(\text{COONa})_3 \cdot 11\text{H}_2\text{O}$
Sodium diborate	$\text{Na}_2\text{Br}_4\text{O}_7$
Sodium dichlorylsulphamidbenzoate	$\text{C}_6\text{H}_4 \cdot \text{COONa} \cdot \text{SO}_2\text{NCl}_2$
Sodium glycocholate	$\text{NaC}_{26}\text{H}_{42}\text{NO}_6$
Sodium oleate	$\text{NaC}_{18}\text{H}_{33}\text{O}_2$
Sodium palmitate	$\text{NaC}_{16}\text{H}_{31}\text{O}_2$
Sodium taurocholate	$\text{NaC}_{24}\text{H}_{44}\text{NSO}_7$
Sodium tartrate	$(\text{CHOH} \cdot \text{COONa})_2 \cdot 2\text{H}_2\text{O}$
Succinic acid	$\text{C}_2\text{H}_4(\text{COOH})_2$

A less complete investigation was made of the following:

Acetone	$(\text{CH}_3)_2\text{CO}$
Aconitine nitrate	$\text{C}_{34}\text{H}_{46}\text{NO}_{11} \cdot \text{HNO}_3$
Copper acetate	$\text{Cu}(\text{CH}_3\text{COO})_2$
Pilocarpine nitrate	$\text{C}_{11}\text{H}_{16}\text{O}_2\text{N}_2 \cdot \text{HNO}_3$
Potassium bichromate	$\text{K}_2\text{Cr}_2\text{O}_7$
Sodium hydroxide	$\text{NaOH}$
Strychnine hydrochloride	$\text{C}_{21}\text{H}_{28}\text{N}_2\text{O}_2 \cdot \text{HCl} \cdot \frac{1}{2}\text{H}_2\text{O}$
Sulphuric acid	$\text{H}_2\text{SO}_4$
Tannic acid	$\text{C}_{14}\text{H}_{10}\text{O}_9 \cdot 2\text{H}_2\text{O}$



## TECHNIQUE.

The technique employed is described here in great detail, because it is hoped that it may be accepted as a standard technique for grading the spermicidal powers of pure substances. The technique enables one to place all substances in "grades" of spermicidal power. The substances in each grade are, on the average, twice as spermicidal as the substances in the next lower grade.

It would have been very convenient if it had been possible to test the effect of each substance dissolved in distilled water; but since distilled water kills sperms by osmosis, it was necessary to dissolve each substance in a fluid isotonic with the sperms. 0.9 per cent. sodium chloride solution is not suitable by itself, for sperms are not very active in it. I made a special investigation (Baker, 1930*a*) to find in what fluid guinea-pig sperms are most active, and elaborated a fluid called Buffered Glucose-Saline, which will be called B.G.S. for short throughout this paper. This fluid has the following composition:

Acid potassium phosphate ( $\text{KH}_2\text{PO}_4$ )	...	0.03	grm. (Dissolve this first)
Sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ )	...	0.6	"
Sodium chloride	...	0.2	"
Glucose	...	3.0	"
Distilled water	...	100	c.c.

The sperm suspensions were made in B.G.S. throughout this investigation (except when acids were being studied), and the various substances tested for spermicidal powers were dissolved in 0.9 per cent. sodium chloride solution. The sperm suspension in B.G.S. was added to an equal quantity of 0.9 per cent. sodium chloride solution containing a definite amount of the substance under investigation. The sperms were thus finally suspended in a solution containing equal parts of B.G.S. and 0.9 sodium chloride solution, together with a known amount of the substance investigated. The control sperms were in a solution of equal parts of B.G.S. and 0.9 per cent. sodium chloride, which is so effective a fluid that the control sperms showed a high degree of activity in nearly every experiment. Those who have only used ordinary saline or Ringer's or Locke's solution would be surprised at the extreme activity of guinea-pig sperms when suspended at 37° C. in B.G.S. or B.G.S. mixed in equal quantities with saline. This laborious investigation, which has occupied a large proportion of my time over a period of nearly two years, could never have been carried through if I could not have relied upon having active control sperms as a matter of course. Controls were invariably examined in every experiment.

The substances themselves were not dissolved directly in B.G.S. for two reasons. Firstly, prolonged contact might cause a reaction between the substance and the phosphates or the glucose of the fluid. Secondly, moulds tend to grow after a time in B.G.S., and a great waste of time would have been involved in making up all solutions afresh from this cause.

Although this technique is effective for the great majority of substances, there are some which present difficulties. Silver salts cannot be tested by it, since silver chloride is at once precipitated. Salts of metals other than potas-

sium and sodium present the difficulty that their phosphates are insoluble, and therefore they are partly precipitated when their solution is added to B.G.S. It is possible to omit the phosphates in these cases, but one must be careful not to enter the results of the experiment if the control sperms are inactive as a result of the omission. The amount of phosphates in B.G.S. is not great enough, however, to render the usual technique inapplicable to these salts. A different fluid takes the place of B.G.S. when acids are being tested, as will be explained later.

The substances are dissolved in 0.9 per cent. saline at various concentrations in the series 4, 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$  per cent., etc. To test a substance at 1 per cent., for instance, a 2 per cent. solution must be used, since it will be mixed with an equal volume of B.G.S. The lowest concentration in this series which kills all sperms in half an hour in four consecutive experiments is determined for each substance.

The glass pipettes, bottles, capsules, specimen tubes, and microscopical slides and coverslips used in the experiments are kept clean but not sterile. Sterility is unnecessary, since sperms are adapted to the non-sterile conditions of the vagina. Pipettes and bottles are cleaned with distilled water. The rest of the glassware is thoroughly washed in tap-water and dried with clean cloths.

The following is a detailed description of the technique.

(1) A thermostat is maintained at 37° C. It contains coverslips, pipettes, and a damp chamber (Fig. 1). The latter is of glass, with a glass lid sealed

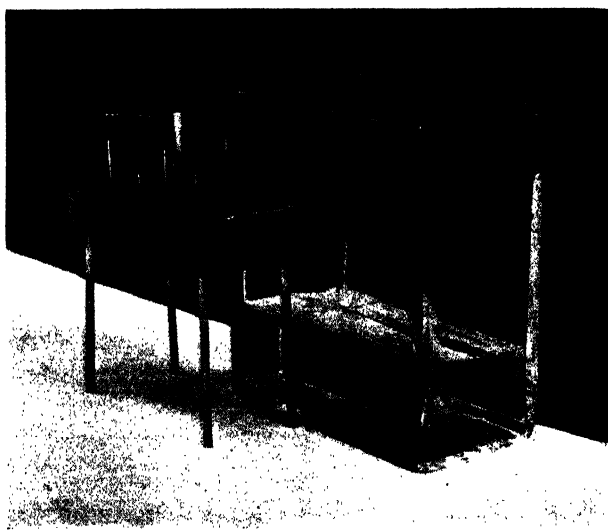


Fig. 1. The damp chamber, with rack containing specimen tubes which fits inside it.

with vaseline to prevent evaporation. There is an inch or two of water in it, containing a crystal of thymol to prevent the growth of moulds. The damp chamber contains small specimen tubes in a wooden rack, which is held out

of the water on four legs. Sperm suspensions will be introduced into the tubes later in the experiment. Since no evaporation can take place in the saturated atmosphere of the damp chamber, the tubes are not corked, and therefore the sperm suspensions do not lack oxygen nor accumulate carbon dioxide. Specimen tubes are preferred to test tubes, since their flat bottoms prevent the sperms from becoming too much aggregated together by gravity.

(2) A thermostatic heater is regulated to deliver a constant stream of water at about 37° C., which passes through a hollow microscope stage. This will prevent the sperms from becoming torpid through cold during examination under the microscope.

(3) The substance to be investigated is dissolved in 0.9 per cent. sodium chloride solution, generally at a concentration of 2 per cent. for the first trial, if the substance is sufficiently soluble.

(4) 0.5 c.c. of 0.9 per cent. sodium chloride solution is introduced into one of the specimen tubes in the damp chamber by means of a graduated pipette. This is the control tube.

(5) 0.5 c.c. of the solution made up in section 3 is introduced into another specimen tube. This is the experimental tube.

(6) An adult male guinea-pig is killed by a blow on the head, and the fur damped in the pelvic region to prevent hairs from getting in the way.

(7) 3 c.c. of B.G.S. are placed in a glass capsule.

(8) The tails of both epididymes of the guinea-pig are cut out and placed in the B.G.S.

(9) Each is cut across once with scissors. Each fragment is pressed several times with flat-pointed forceps to cause the sperms to come out. The fragments are then removed and thrown away.

(10) The fluid is sucked into and out of a pipette to break up any clumps of sperms and form an even suspension.

(11) 0.5 c.c. of the sperm suspension is transferred to each of two empty specimen tubes in the damp chamber in the thermostat.

(12) The fluids are left to warm up.

(13) After a quarter of an hour the sperm suspension is transferred with a pipette from one of the tubes containing it to the control tube. The control tube now contains sperms suspended in a mixture of B.G.S. and saline in equal quantities.

(14) Air is bubbled three times through the contents of the control tube with a pipette. This mixes the fluids and promotes respiration by the sperms.

(15) The sperm suspension in the other tube is transferred to the experimental tube. The latter now contains sperms in a mixture of B.G.S. and saline in equal quantities, together with the substance under investigation at a concentration of 1 per cent., if it has been made up, according to section 3, at 2 per cent.

(16) Air is bubbled three times through the contents of the experimental tube.

(17) The time is recorded at the start of the experiment.

(18) Two hollowed microscopical slides are labelled with a grease-pencil. One is labelled C (control), the other with letters denoting the name of the substance under investigation.

(19) Over the grease-pencil lettering on each slide is fixed a blank gummed-paper label, stuck down at one side in such a way that it may be turned aside to disclose the grease-pencil lettering. The object of this arrangement is explained in section 23.

(20) The labelled slides are placed on the floor of the thermostat.

(21) Twenty-five minutes after the start of the experiment, air is bubbled through the control tube, and with the same pipette three drops of the fluid are transferred to the hollow of the slide marked C. A warm coverslip is applied. Three drops of fluid do not fill the hollow of the slide. A large bubble of air is included below the coverslip, which prevents the sperms from becoming inactive quickly owing to inability to respire. The slide is left on the floor of the thermostat.

(22) The process described in the last section is applied to the contents of the experimental tube and to the slide labelled with letters denoting the name of the substance under investigation.

(23) The slides are shuffled together till the observer does not know which is which. Bias, conscious or unconscious, is thus avoided. The shuffling is particularly valuable when two or three substances are being tested at the same time. (See section 38.)

(24) As exactly as possible half an hour after the start of the experiment (section 17), the slides are removed one by one from the thermostat and examined under the microscope with a  $\frac{1}{8}$  in. objective and No. 2 eyepiece, on the hot stage mentioned in section 2.

(25) The activity of the sperms is recorded on the blank labels on the slides, as follows:

III indicates that the majority of the sperms are moderately or very active.

II indicates that 10 per cent. of the sperms are moderately active, *or* that the majority are feebly active, *or* that there is any greater amount of activity that is less than III.

I indicates any activity less than II, including the slightest movement in a single sperm.

0 indicates that examination of ten microscopical fields fails to reveal the slightest movement in a single sperm. If the observer has seen no movement with certainty, but thinks it possible that a single sperm has moved, he starts again and begins to examine ten more microscopical fields.

Currents in the suspension, caused by movement of the coverslip, sometimes give a deceptive appearance against which the observer must guard.

The following sign is occasionally useful:

III + indicates extreme activity.

The following signs are used to prevent the delay which would arise from prolonged indecision:

II + indicates that it cannot be quickly decided whether the sperms should be graded as II or III.

I + indicates that it cannot be quickly decided whether the sperms should be graded as I or II.

It is usually possible to place sperms in grades III or II in a few seconds. Grade 0 takes up to a minute, since ten fields must be examined, and grade I may take as long, if the first active sperm is seen in the tenth field.

At first sight this method of grading seems complicated and arbitrary, but experience proves it to be quick and reliable. It is very important that the grading should be done quickly, in order that the slides may be examined as exactly as possible half-an-hour after the start of the experiment. Quickness is of course particularly important when several substances are being examined at the same time.

(26) The blank labels are turned aside to disclose the identity of the slides.

(27) If the control sperms show an activity of III or III +, the result of the experiment is recorded.

(28) If the control sperms show an activity of less than III, the result is not recorded. This only happens when an immature or diseased guinea-pig is used.

(29) If the activity of experimental sperms is reduced to 0, the experiment is repeated with the substance at half the concentration used before. It is repeated again and again if necessary, at lower and lower concentrations in the series 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$  per cent., etc., until active sperms are found.

(30) If the activity of the experimental sperms is not reduced to 0, the experiment is repeated at double the concentration used before. It is repeated again and again, if necessary, at higher concentrations in the series  $\frac{1}{2}$ , 1 and 2 per cent. 2 per cent. is not exceeded.

(31) The lowest concentration which suffices to kill all sperms is thus ascertained, and the experiment is performed again at this concentration.

(32) If all sperms are killed, the experiment is performed a third time at this concentration.

(33) If all sperms are killed, the experiment is performed a fourth time at this concentration.

(34) If all sperms are killed, this concentration is recorded as the "killing concentration." The killing concentration is the lowest concentration in the

series 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$  per cent., etc., which reduces the activity of the sperms to 0 in four consecutive experiments.

(35) If in any of the experiments of sections 31, 32 and 33 all the sperms are not killed, the next higher concentration must be tried until the killing concentration, defined in section 34, is found.

(36) All substances whose killing concentration is the same are placed in the same "grade" of spermicidal power. If the killing concentration is  $\frac{1}{2^x}$ , then the substance is said to be in "grade  $x$ ." Thus sodium oleate always kills sperms at  $\frac{1}{32}$  per cent., but not at  $\frac{1}{64}$  per cent. Its killing concentration is  $\frac{1}{32}$  per cent. or  $\frac{1}{2^5}$ . Sodium oleate therefore falls into grade 5. It is obvious that substances in each grade have, on the average, twice the spermicidal power of the substances in the next lower grade.

(37) The experiment is performed three times with each substance at half the killing concentration. This enables the observer to compare the spermicidal powers of substances which fall into the same grade.

(38) From one to three different substances may be tested at the same time against the same control. A single substance must never be tested more than once at the same concentration against the same control.

(39) The technique described above is suitable for most substances except acids. Hydrogen-ions themselves immobilise sperms, irrespective of what the acid may be. (See below, under "Acidity.") If the experiments with acids were carried out in the way described above, one might simply be measuring what concentration of each acid is sufficient to overcome the sodium hydrogen phosphate buffer in the B.G.S. A fluid containing so much sodium hydrogen phosphate is clearly unsuitable for an investigation of the spermicidal powers of acids.

A neutral fluid is therefore used while acids are being tested, instead of the alkaline B.G.S. This neutral fluid is made as follows. 6 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  are dissolved in 100 c.c. of water, and this solution is added to 5.2 per cent. glucose solution until a sample of it gives a yellowish-green reaction with the "Universal" Indicator of British Drug Houses. A definite formula cannot be given for this fluid, since the acidity of glucose solutions varies.

#### RESULTS OF THE MAIN INVESTIGATION.

Table I gives the whole of the results of this investigation, except a few inconsistent results which must have been caused by mistakes in diluting the substances to the various concentrations. In such an extensive investigation as this it was perhaps to be expected that an occasional slip would be made. When an apparently inconsistent result was obtained, experiments were performed to find whether the inconsistency was apparent or real. If the result was shown to have been due to a mistake, neither the inconsistent result



nor the experiments performed to test the inconsistency are recorded in the table. Thirteen inconsistent results were obtained, mostly towards the beginning of the investigation. Twenty extra experiments were performed, which showed these results to be due to mistakes. Table I shows the 292 consistent results obtained in the investigation.

The following is an abstract of Table I. In each grade the substances are arranged in the order of their spermicidal powers, as determined by the results of the experiments at half the killing concentration, or in some cases at one-quarter the killing concentration. The order within each grade cannot be regarded as final.

*Grade 8.* (Killing concentration  $\frac{1}{256}$  per cent.)

Mercuric chloride	Formaldehyde
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*Grade 6.* (Killing concentration  $\frac{1}{64}$  per cent.)

Saponine	Hexyl resorcin	Iodine
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*Grade 5.* (Killing concentration  $\frac{1}{32}$  per cent.)

Acetic acid	Sodium oleate
Succinic acid	Sodium palmitate

*Grade 4.* (Killing concentration  $\frac{1}{16}$  per cent.)

Lactic acid	Potassium permanganate	Citric acid
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*Grade 2.* (Killing concentration  $\frac{1}{4}$  per cent.)

Sodium taurocholeate	Quinine urea hydrochloride
Potassium aluminium sulphate	Cresol
Sodium glycocholeate	Potassium borotartrate
Dioxyquinolin sulphate	

*Grade 1.* (Killing concentration  $\frac{1}{2}$  per cent.)

Quinine hydrochloride	Quinine bisulphate
Potassium oxyquinolin sulphate (Chinosol)	Phenol
Potassium cyanide	Hydrocyanic acid

*Grade - 1.* (Killing concentration 2 per cent.)

Potassium capronate

*Ungraded.* (These fail to kill at 2 per cent.)

Boric acid	Sodium tartrate
Ammonium chloride	Magnesium sulphate
Potassium butyrate	Sodium diborate
Sodium citrate	Ethyl alcohol
Hexamine	Sodium dichlorylsulphamidbenzoate

These substances will now be considered in the order of their spermicidal powers.

*Mercuric chloride.* This appears to be the most spermicidal substance that exists. In an attempt to find whether any other substance surpassed it, four



substances which were not included in the main investigation were tried at  $\frac{1}{312}$  per cent., to find whether they had any effect at all at this concentration. Table I shows that mercuric chloride has considerable effect at  $\frac{1}{312}$  per cent. These substances were aconitine nitrate, pilocarpine nitrate, sulphuric acid and sodium hydroxide. The two former seemed to have a slightly stimulating effect on the sperms at this concentration, when compared with the controls, though this cannot be regarded as certain. The two latter substances were without effect. The extreme spermicidal power of mercuric chloride is of academic rather than practical interest, for no one would include such a poisonous substance in a pessary. It presumably kills by forming mercury albuminates with the proteins of the sperms.

*Formaldehyde.* The solutions were made up on the assumption that the "formalin" used was actually 40 per cent. formaldehyde. Probably the concentration was slightly less, so that formaldehyde may be slightly more spermicidal than the experiments show. Anyhow, it runs mercuric chloride very close. Dr H. M. Carleton has made some experiments with it, and finds that it is without effect upon the vagina or uterus of the rabbit when used at concentrations far greater than the killing concentration. I wish to thank Dr Carleton for allowing me to mention this unpublished result. A weak solution of formalin would probably be an effective contraceptive if used as a vaginal douche. One cannot detect formaldehyde if a solution at the killing concentration is taken into the mouth and swallowed. The preparation of a solid pessary containing or producing formaldehyde presents difficulties, which are referred to under the heading "Hexamine."

*Saponine.* This poisonous glucoside was suggested by Prof. J. B. S. Haldane on account of its power of reducing surface tension. Unless experiments prove the contrary, it may be suspected of being too irritant for use as a contraceptive. Powdered quillaia bark is contained in the Monsol pessary.

*Hexyl resorcin.* This substance, sometimes known as Caprokol, was tried on account of its very great power of reducing surface tension. Of all the substances tried it seems the most likely to be useful as a contraceptive, though no experiments have yet been made to find whether it is harmless. It is thirty-two times as spermicidal as quinine bisulphate or chinosol. It is not very soluble, but this may in a sense be an advantage, for with very soluble substances there is always the possibility of dangerously high concentrations occurring in the vagina if there is very little vaginal fluid.

*Iodine.* This was dissolved with four times its weight of potassium iodide. It would probably be too irritant for practical use.

*Acetic acid.* Diluted vinegar is said by Stopes (1925) to be widely used as a contraceptive. It has not, to my knowledge, been incorporated in a solid pessary (as lactic acid has). It suffers from the same disadvantage as other acids, namely that semen is alkaline, and that this alkalinity must be neutralised before the sperms are affected. The vaginal fluid is also said to be alkaline during sexual excitement. It is interesting to notice that a monobasic acid

is the most spermicidal. It had been suggested to me that dibasic acids would be more effective than monobasic, and tribasic than dibasic. This is not so.

*Succinic acid* is dibasic. It has almost exactly the same spermicidal powers as acetic acid. It belongs to the poisonous oxalic acid series, and is only significant for comparative purposes.

*Sodium oleate*. Prof. J. B. S. Haldane suggested soaps, on account of their power of reducing surface tension. His expectations were fully realised, for sodium oleate kills at  $\frac{1}{32}$  per cent. It is therefore sixteen times as spermicidal as quinine bisulphate or chinosol, which are so commonly used in pessaries. Its effect upon sperms is not due to the alkalinity of the solution, for neutralised solutions kill at the same concentration. This was proved by a series of experiments in which the  $\frac{1}{16}$  per cent. sodium oleate solution was neutralised with lactic acid before it was mixed with an equal quantity of sperm suspension in B.G.S. The 0.9 per cent. sodium chloride solution in the control tube was acidified by the same amount of lactic acid, lest it might be thought that the inactivity of the experimental sperms was due to the lactic acid itself, and not to the sodium oleate. In all other respects the experiments were carried out as usual. The following results were obtained in four consecutive experiments:

Experimental sperms (Sodium oleate $\frac{1}{32}$ %, previously neutralised)				Corresponding controls			
0	0	0	0	III +	III	III	III

It is clear that it is not the alkalinity of sodium oleate solutions that causes them to be so spermicidal.

I found that sodium oleate is also very spermicidal with human sperms, though less so than with guinea-pig ones. (See Potassium capronate.) I therefore prepared a number of pessaries with various soaps in gelatine gels, with and without the addition of glycerine, and these seemed likely to be far more efficient than any existing pessary. Dr Carleton kindly allows me to say that he finds that these pessaries are harmful to the vagina and uterus of the rabbit. Weak soap solutions could probably be used with advantage as contraceptive douches.

The great spermicidal power of soap raises some interesting points. No woman who was desperately anxious to have a baby would soak herself (vagina included) in a weak solution of quinine or chinosol shortly before coition; yet she would have no hesitation in soaking herself in a soap solution in a hot bath, although soap is very much more spermicidal than quinine or chinosol. Bath water certainly enters the vagina in the case of women who have borne children. It seems to be conceivable that the fall in the birth rate among the wealthier classes during the latter half of the nineteenth century may be correlated with the installation of proper hot water systems in the houses of the rich at that time. Another interesting point is that occlusive pessaries made of rubber are often placed in soapy water before introduction into the vagina, so that they may slip in easily. Their efficacy no doubt depends largely on the soap.

Sodium oleate and palmitate are slightly more spermicidal than appears from the table, for the solutions were made up on the assumption that the samples used did not contain water.

Dr M. Stopes and Mrs Margaret Sanger inform me that soap-suds have been used as a contraceptive by prostitutes and others.

*Sodium palmitate* has almost exactly the same spermicidal power as the oleate. It is less soluble.

*Lactic acid* has the advantage that it is normally present in the vaginal fluid. The solutions were made up in such a way that 100 c.c. of a  $\frac{1}{2}$  per cent. solution contained  $\frac{1}{2}$  grm. of actual lactic acid, not  $\frac{1}{2}$  grm. of the 75 per cent. solution sold as lactic acid. This monobasic acid has almost exactly the same spermicidal power as the tribasic citric acid. It is open to the same objection as other acids. (See Acetic acid.) It is sold in the form of a cocoa-butter pessary. It is said by Stopes (1925) to be used also as a douche.

*Potassium permanganate* is said by Stopes (1925) to be used as a douche at  $\frac{1}{20}$  per cent. Its killing concentration with guinea-pig sperms is  $\frac{1}{10}$  per cent., but very few sperms remain alive at  $\frac{1}{20}$  per cent.

*Citric acid* is said by Stopes (1925) to be used in douches. It is tribasic, but less spermicidal than the monobasic acetic acid. It is open to the same objection as other acids. (See Acetic acid.)

*Sodium taurocholate*. The bile salts were suggested by Prof. Haldane on account of their power of reducing surface tension. They are not very powerful spermicides. Possibly they will be found useful in conjunction with other more spermicidal substances.

*Potassium aluminium sulphate*. This was made up in such a way that 100 c.c. of a 1 per cent. solution contained 1 grm. of the anhydrous salt. Alum is said by Stopes (1925) to be mentioned as a contraceptive in Sanskrit writings. It is a constituent of the foaming pessary finil. Perhaps its astringent effect on mucous membranes may render it rather unsuitable for continued use, but this has not been proved.

*Sodium glycocholate*. See Sodium taurocholate, above.

*Dioxyquinolin sulphate* is a constituent of the foaming pessaries, semori and finil.

*Quinine urea hydrochloride* is a constituent of a very small pessary made by Messrs Parke, Davis and Co. The only other constituent is sodium chloride. It is not known whether it has the disadvantage of having an appreciable anaesthetizing effect on the vagina.

*Cresol*. Monsol fluid, which enters into the composition of the monsol pessary, contains substances allied to cresol.

*Potassium borotartrate* is a constituent of semori.

*Quinine hydrochloride*. This was dissolved in such a way that 100 c.c. of a 1 per cent. solution contained 1 grm. of the salt as one buys it, with two molecules of water.

*Potassium oxyquinolin sulphate*. There is some doubt whether chinosol can

be regarded as a pure substance. Martindale (1928) regards it not as a double sulphate of potassium and oxyquinolin, but as a mixture, in varying proportions, of potassium sulphate and dioxyquinolin sulphate. It is sold in the form of a cocoa-butter pessary by Messrs Lambert.

*Potassium cyanide.* This was tested merely for comparison with other substances. It is remarkable that such a poisonous substance should only have one-sixteenth the spermicidal power of soap. It was dissolved in such a way that 100 c.c. of a 1 per cent. solution contained 1 gram. of the actual salt, not of the 95 per cent. pure substance as one buys it.

*Quinine bisulphate* has probably been as much used as any substance as a chemical contraceptive. It is sold in the form of a cocoa-butter pessary by Messrs Lambert. In this investigation it was dissolved in such a way that 100 c.c. of a 1 per cent. solution contained 1 gram. of the salt as one buys it, with seven molecules of water. Only 59 per cent. of this salt is alkaloid. In quinine hydrochloride 82 per cent. is alkaloid. It is therefore remarkable that the bisulphate is nearly as spermicidal as the hydrochloride. In view of the widespread distrust of chemical contraceptives used by themselves, the low spermicidal power of the substance most commonly used is significant.

*Phenol* is less spermicidal than one would expect from its germicidal power. It was tested by the ordinary technique, not by the modification for acids.

*Hydrocyanic acid.* The same remarks apply to prussic acid as to potassium cyanide. The solution was brought at 2 per cent. in water, and 0.9 per cent. of sodium chloride added.

*Potassium capronate.* When the high spermicidal power of sodium oleate on guinea-pig sperms was discovered, it was tried on human semen. Although effective, it was less so than on guinea-pig sperms. It appeared probable that its reduced power was due to the large calcium content of human semen, which caused the precipitation of the insoluble calcium oleate. For this reason lower members of the acetic acid series were chosen instead of the oleic, on account of the solubility of their calcium soaps. Pessaries containing the potassium and sodium soaps of coconut oil were prepared for the same reason. Unfortunately the capronate has slight spermicidal powers, and the butyrate still less.

*Boric acid.* This antiseptic has surprisingly little spermicidal power. It is a constituent of finil.

*Ammonium chloride* was tried on account of the ease with which it penetrates cell membranes.

*Potassium butyrate.* See Potassium capronate.

*Sodium citrate.* It was thought that the salts of tribasic acids might be effective.

*Hexamine.* It was thought that hexamine might be a convenient producer of formaldehyde. Unfortunately it produces formaldehyde very slowly, especially in alkaline solutions, and is scarcely spermicidal.

*Sodium tartrate* is a product of the interaction of the foam-producers in effervescing pessaries.

*Magnesium sulphate* is the chief ingredient of the very large pessary called "contraps."

*Sodium diborate*. Borax has scarcely any effect on sperms.

*Ethyl alcohol* is almost without effect at 2 per cent.

*Sodium dichlorylsulphamidbenzoate* is the least spermicidal of all the substances tried. It is supposed by the makers to be the essential constituent of speton, but actually the spermicidal powers of that pessary must be due to the carbon dioxide produced by the tartaric acid and sodium bicarbonate. Perhaps sodium dichlorylsulphamidbenzoate would be found to be more spermicidal if it were dissolved directly before the experiment. In a paper distributed by the manufacturers, Prof. Kionka concludes that it must be a good spermicide because he has found it to be germicidal.

#### NOTES ON OTHER SUBSTANCES.

A few experiments were made on other substances, but not sufficient for grading purposes.

Copper acetate reduces the activity of sperms, but not to 0, at  $\frac{1}{32}$  per cent. At this concentration potassium bichromate, acetone and tannin acid have no effect. Strychnine hydrochloride has no effect even at 1 per cent. It cannot be tested at 2 per cent., because it will not dissolve at 4 per cent. I have never seen sperms so active as with strychnine hydrochloride at  $\frac{1}{8}$  per cent., but I have not made sufficient experiments to prove that strychnine actually activates them at this concentration. If so, practical use might be made of this knowledge in cases where infertility results from the inactivity of the sperms. A very small quantity of strychnine hydrochloride, dissolved in B.G.S., might be added to semen caught in a sheath. The semen could then be inserted in the vagina or uterus.

Aconitine nitrate, pilocarpine nitrate, sulphuric acid and sodium hydroxide are mentioned above under the heading "Mercuric chloride."

#### FOAMING MIXTURES.

Effervescing pessaries (semori, finil and speton) contain tartaric acid and sodium bicarbonate, which react in the vagina to form a foam of bubbles of carbon dioxide, whose purpose it is to spread the spermicidal substance or substances to all parts. Now carbon dioxide has itself the power of immobilising sperms, and I therefore decided to test foaming mixtures by the usual technique. The mixtures were dissolved in such a way that 100 c.c. of a 1 per cent. solution contain 1 gm. of a mixture of tartaric (or citric) acid and sodium bicarbonate in the appropriate proportions. The sodium tartrate (or citrate) formed is probably partly responsible for the results obtained.

The only differences from the usual technique were the following:

- (1) 0.5 c.c. of sperm suspension in B.G.S. is placed in each of two specimen tubes in the damp chamber in the thermostat and 0.5 c.c. of 0.9 per cent. sodium chloride solution is added to it.
- (2) The experimental tube, containing a weighed amount of dry foaming mixture is placed in the thermostat, not in the damp chamber.
- (3) It is transferred to the damp chamber immediately before the start of the experiment.
- (4) At the start of the experiment, 1 c.c. of sperm suspension is transferred with a pipette from one of the tubes containing it to the experimental tube. The other tube containing sperm suspension is the control tube.

The following results were obtained. The control sperms showed an activity of III + or III in each experiment.

	8 %				4 %			2 %		1 %
Tartaric foaming mixture	0	0	0	0	I	I	I	II+	II+	II+
Citric foaming mixture	0	0	0	0	I	I	I	I	I+	I+

Each mixture therefore kills at 8 per cent. every time. Now supposing that 7.5 c.c. of fluid are present in the human vagina after coition, and that human sperms behave in the same way as those of the guinea-pig to the products of foaming mixtures, it is clear that 0.6 gm. of foaming mixture would suffice to immobilise all the sperms present. One of the commercial foaming pessaries contains more than this amount.

Since the foaming mixtures only kill at 8 per cent., their spermicidal powers are really very low; but one can safely introduce very much more of them into the vagina than one can of most spermicidal substances.

I shall undertake experiments to find whether the products of these foaming mixtures actually kill or only temporarily immobilise sperms. It seems probable that a pessary consisting simply of about a gram of foaming mixture would be much more spermicidal than many non-foaming pessaries on the market. The presence of an excess of acid would probably increase the spermicidal power.

#### THE EFFECTS OF VARIOUS SUBSTANCES ON THE BEHAVIOUR AND STRUCTURE OF SPERMS.

Experiments were performed in order to find out in what way some of the substances investigated kill sperms. Six substances were investigated from this point of view, namely formaldehyde, hexyl resorcin, sodium oleate, lactic acid, dioxyquinolin sulphate and quinine bisulphate. It was thought unnecessary to extend this study to all the substances whose spermicidal powers had been investigated.

Unfortunately I could find in the literature no accurate description of the structure of the head of the guinea-pig sperm. The best is that of Meves (1899),

but he gives figures of transverse sections of the head which do not correspond with his surface views. I had therefore first to study the structure of the normal guinea-pig sperm. (See Fig. 2.)

The nucleus is shaped like the bowl of a spoon. Meves calls the convexity of the bowl "dorsal" and the concavity "ventral," and indeed sperms seem usually to come to rest with the convexity uppermost, though in swimming the progression is spiral. The acrosome is also somewhat spoon-shaped, but with the convexity "ventral." Its lip is recurved. It is attached to the front end of the nucleus, which it is said by Meves and all other workers to ensheath. So far as I can make out, this is inaccurate. The acrosome seems to me only to cover the nucleus to any extent *ventrally*. Dorsally it only covers the extreme anterior end of the nucleus. The line of the posterior end of the acrosome is quite easily seen crossing the nucleus, and it has been supposed that the acrosome extends to this line both above and below the nucleus. Very careful focussing has made me almost certain that this line is only seen by transparency when the sperm is viewed from "above."

Fig. 2 was drawn from numerous observations upon fresh sperms. The section B is an optical section. B and C are to be regarded as expressions of my opinion as to the structure of the acrosome, and not as pictures of what can be seen in a single sperm viewed from the side. The backward extent of the acrosome below the nucleus can only be determined in ventral or dorsal view.

In Figs. 2 and 3 the nucleus, where seen in optical section, is marked with parallel straight lines. The middle piece is dotted.

Two series of experiments were performed to find the effects of the six substances mentioned on the behaviour and structure of sperms.

In one series the experiments were carried out as usual up to section 16, the substance being present at *half* the concentration which always kills in half an hour. Directly the sperm suspension had been added to the solution of the substance investigated, a slide was prepared and examined forthwith under the  $\frac{1}{4}$  in. objective on the hot stage, and at intervals up to a quarter of

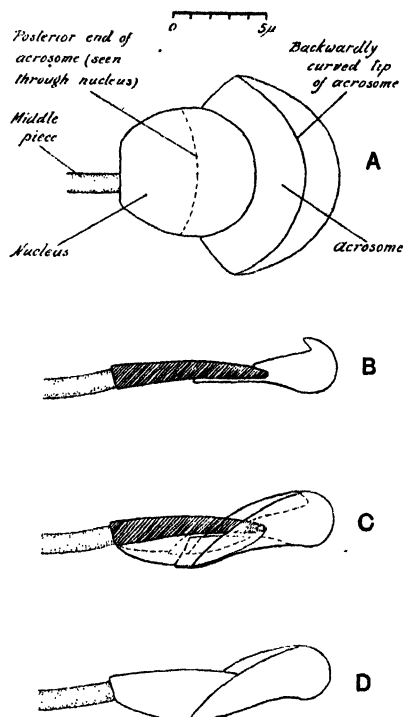


Fig. 2. Head of normal guinea-pig sperm. A. "Dorsal" view. B. Longitudinal section. C. Viewed from the side as a transparent object. D. Viewed from the side as an opaque object.

an hour later. The movements of those sperms which were active were carefully observed, to find whether special types of movement characterised the various substances. Controls were always examined.

In the other series the experiments were carried out as usual up to section 17, the substance being present at *twice* the concentration which always kills in half an hour. A flat microscopical slide was prepared half an hour after the addition of the sperm suspension to the solution of the substance investigated.

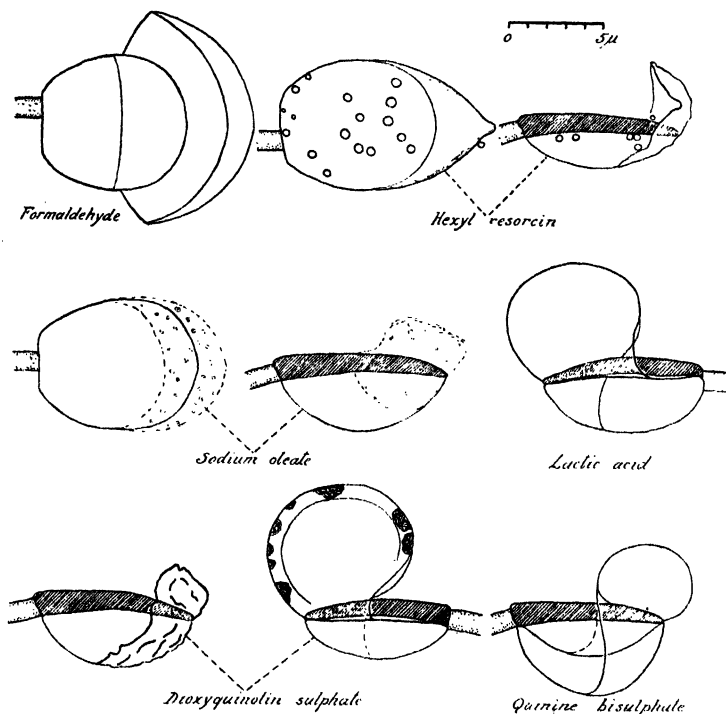


Fig. 3. Heads of guinea-pig sperms killed by various substances at twice the killing concentration.

The sperms were then subjected to close examination under an oil-immersion objective, to find what structural alterations, if any, had taken place in them. Controls were always examined. Some of the sperms were also fixed in a chromosium fixative after the substance had acted for half an hour, and permanent preparations were made and studied.

In what follows each of the six substances is dealt with separately. Under each heading the results of both series of experiments are recorded. The reader is referred to Fig. 3 for representations of sperms acted upon by the six substances studied at twice the killing concentration.

**Formaldehyde.** At half the killing concentration some of the sperms show spiral progression, probably on account of the shape of the acrosome not being



affected by formaldehyde. Other sperms oscillate rapidly. By oscillation is meant repeated small to-and-fro movements of the head and tail without progression. At twice the killing concentration there is no visible effect upon the sperm, which resembles a living one. This is the only substance among the six which does not distort the acrosome.

*Hexyl resorcin.* Since very few sperms remain active at half the killing concentration, hexyl resorcin was tried at one-quarter the concentration instead. No characteristic behaviour on the part of the sperms could be detected, some behaving in one way and some in another. At twice the killing concentration the acrosome is shrunk. Its outline is distinct, but often contorted. Large refringent granules are scattered over the nucleus, possibly derived from the substance of the internal part of the acrosome. It seems probable that hexyl resorcin causes the acrosome to swell up and burst, for at one-quarter of the killing concentration the acrosome of those sperms which are dead is commonly swollen. The shrunk acrosome seen at twice the killing concentration is probably the collapsed remnant.

*Sodium oleate.* Rapid oscillation through a small distance is characteristic of those sperms which are not killed outright by sodium oleate at half the killing concentration. At twice the killing concentration the acrosome is very much shrunk and transparent, and lacks a clear outline. It appears to contain small, indistinct granules. As with hexyl resorcin, it has apparently swollen up and burst, for swollen acrosomes are seen at half the killing concentration.

*Lactic acid.* At half the killing concentration, the tails of the active sperms tend to curl up slowly and then straighten out quickly, resulting in slow, non-spiral movement in the arc of a small circle. The concavity of the curved tail is directed towards the centre of the circle. At twice the killing concentration the acrosome is swollen into a rounded structure situated on the dorsal surface of the nucleus, roughly circular in optical section when viewed from the side.

*Dioxyquinolin sulphate.* At half the killing concentration quite active lashing movements of the tail occur, but very few sperms progress spirally. There is no oscillation. At twice the killing concentration the acrosome is commonly swollen up into a bladder situated dorsally. The bladder is thick-walled and the walls have opaque patches on them. In many sperms it appears that the acrosome has swollen up into a bladder which has then burst. In these the acrosome is shrunk and distorted. Its outline, which remains very distinct, is puckered.

*Quinine bisulphate.* At half the killing concentration those sperms which are active tend to oscillate rapidly through a very short distance without any progression. There are no large bending movements of the tail. At twice the killing concentration the acrosome is swollen up into a rounded structure on the dorsal surface of the front end of the nucleus, similar to that produced by lactic acid. Sometimes it appears to have burst, for the acrosome is reduced to a small granular mass capping the anterior end of the nucleus.

Careful measurements fail to reveal any swelling or shrinkage of the nucleus

as a result of the action of any of these substances, nor is the middle-piece visibly affected. The tail is often rather strongly curved just behind the middle-piece in sperms subjected to the action of dioxiquinolin sulphate and quinine bisulphate, but this is not to be regarded with certainty as a characteristic reaction. It is the acrosome which is the most vulnerable part of the sperm, so far as structural changes are concerned. Prof. G. T. Popa, of the Histological Laboratory at Jassy, Rumania, has arrived at this conclusion quite independently in a large investigation of the biology of the sperms of several Mammals. It was my fortune to see a demonstration of his work at the Second International Congress for Sex Research.

### OSMOSIS.

A series of experiments was performed to find to what extent guinea-pig sperms were susceptible to changes in the osmotic pressure of a substance which is not chemically destructive to them. For this purpose sperms were suspended in various concentrations of sodium chloride.

The experiments were carried out as follows. Solutions of sodium chloride were made up at 5, 2, 1.5, 0.9 (control) and 0.5 per cent. 1 c.c. of each was placed in a specimen tube in the usual damp chamber in the thermostat. 1 c.c. of distilled water was placed in another tube in the damp chamber. The fluids were left to warm up. An adult male guinea-pig was killed by a blow on the head, and the tail of one epididymis removed. The tail was cut into four approximately equal portions, of which one was placed in the tube containing 0.9 per cent. sodium chloride, and one or more in one or more of the other tubes. The portions of epididymis were squeezed with forceps and removed. Air was bubbled through the fluids with pipettes. Twenty-five minutes after the introduction of the sperms into the fluids, air was bubbled through again and hollowed slides prepared from the contents of each tube. As exactly as possible half an hour after the introduction of the sperms into the fluids, the slides were examined under the microscope on the hot stage. The experiment was repeated until it had been performed three times at each experimental concentration, and seven times at the control concentration (0.9 per cent.). The control sperms were not very active, on account of the acidity of sodium chloride solutions. The following results were obtained:

0 %	0.5 %	0.9 %	1.5 %	2 %	5 %
0	.	III	.	.	.
0	.	II+	.	I	0
.	.	II+	.	I	0
0	II	II+	I+	.	.
.	.	II+	I	I	0
.	III	III	I	.	.
.	I+	III	.	.	.

The results show that sperms are very susceptible to changes in osmotic pressure. The increase from 0.9 to 1.5 per cent. results in the death of the great majority of the sperms. Acidity cannot be the cause of the inactivity of sperms

at increased concentrations, for 2 per cent. sodium chloride has the same *pH* (about 6.2) as 0.9 per cent. Distilled water always kills every sperm.

No doubt many of those substances tested in the main investigation which only kill at high concentrations do so largely by virtue of their osmotic pressure.

#### ACIDITY.

The reader is referred to the section upon acetic acid, above, for some remarks on acids.

A series of experiments was performed to find the effects of fluids at various *pH* on sperms. Acetic, succinic, citric and lactic acids were investigated. Each was dissolved at various concentrations in a glucose-saline solution containing phosphates, and the *pH* of each solution was determined. The technique was almost precisely the standard one. The *pH* given is that of the fluid without the sperms. The activity of the sperms was determined as usual after half an hour at the temperature of the body. The control sperms showed an activity of III or III + in every case. The following results were obtained:

<i>pH</i> 4.0-4.9	Acetic	0	0	0	0
	Succinic	0	0	0	0
	Citric	0	0	0	0
	Lactic	0	0	0	0
<i>pH</i> 5.0-5.9	Acetic	0	0	I	II
	Succinic	I	I	I +	II +
<i>pH</i> 6.0-6.9	Acetic	III			
	Citric	I	II	III	
	Lactic	II	II	III	

This may be summarised as follows: Sperms are all killed below *pH* 5. At *pH* 5.0-5.9 their activity is usually reduced to about I (less than 10 per cent. moderately active). At *pH* 6.0-6.9 their activity is usually reduced to about II (less than half moderately active).

It is important to note that it is the concentration of hydrogen-ions, and not the nature of the anions, that determines the inactivity of the sperms. It makes little or no difference what acid is used; all have the same effect at the same *pH*. Again, citric acid always kills at  $\frac{1}{16}$  per cent. when tested by the standard technique; but sodium citrate fails to kill at 2 per cent.

An apparent exception to the rule that the anion does not matter is afforded by boric acid. A solution of boric acid at *pH* 6.3 reduced the activity of sperms to I in two experiments. This is not to be interpreted, however, as indicating any special spermicidal power of the boric anion. It is simply that boric acid dissociates very slightly, and therefore the solution at *pH* 6.3 was a very concentrated one, thirty-two times as concentrated as a solution of citric acid at approximately the same *pH*. No doubt the boric acid exerted its effects largely by osmosis.

#### SURFACE TENSION.

The importance of Prof. Haldane's suggestion that substances which lower surface tension would be likely to be good spermicides has already been

stressed. It was this conception which resulted in the discovery of the great spermicidal powers of hexyl resorcin.

I was anxious to find whether there was any direct correlation between the power to reduce surface tension and the power to kill sperms. In order to arrange these substances in the order of their power to reduce surface tension, I dissolved each of them at a concentration of  $\frac{1}{32}$  per cent. in 0.9 per cent. sodium chloride solution. I then transferred each solution drop by drop with a pipette to a measuring cylinder, and counted the number of drops required to make 10 c.c. The same pipette was used for each substance, and it was held vertically throughout.

The observations were made at room temperature, which did not vary greatly. The number of drops is shown below. The last figure is probably insignificant, owing to the lack of precision in the method employed. The substances are arranged in the order of their capacity to reduce surface tension.

	Number of drops	Killing con- centration (%)
Hexyl resorcin	741	$\frac{1}{32}$
Sodium taurocholate	542	$\frac{1}{4}$
Sodium glycocholate	493	$\frac{1}{4}$
Sodium oleate	476	$\frac{1}{32}$
Potassium capronate	438	2
Saponine	410	$\frac{1}{32}$
Sodium palmitate	403	$\frac{1}{32}$
Sodium chloride 0.9 %	385	

It will be observed that there is no direct relation between capacity to reduce surface tension and spermicidal power. The bile salts are very surface-active, but only kill at  $\frac{1}{4}$  per cent.

Substances which reduce surface tension should be included in pessaries, not only because such substances are usually spermicidal, but also because their presence renders it more probable that the smallest crevices in the folds of the vagina will be reached. Perhaps the bile salts will find an application here, used in conjunction with a more spermicidal substance.

#### THE IDEAL CHEMICAL CONTRACEPTIVE.

It appears to me that the ideal chemical contraceptive should have the following characters:

- (1) It should be inexpensive ( $\frac{1}{2}$ d. or less).
- (2) It should be solid, and therefore require no special appliance for introduction into the vagina.
- (3) It should be small (less than 1.5 grm.).
- (4) It should be unaffected by the ordinary range of climate.
- (5) It should leave no trace on the skin when handled.
- (6) It should contain no volatile or odorous substance.
- (7) It should be absolutely non-irritant and harmless to the vagina, uterus and penis. It is possible that chemical contraceptives may sometimes find

their way to the uterus, so the absence of any action on the uterine mucosa must be assured.

(8) It should be without physiological effect if absorbed into the blood stream.

(9) It should form a dense foam of small bubbles within the vagina, to carry the spermicidal substance to all parts.

(10) It should contain a substance which will give strength and permanence to the foam.

(11) It should contain a substance reducing surface tension, not only because such substances are usually good spermicides, but also to ensure the smallest crevices in the folds of the vagina being reached.

(12) It should dissolve completely in 7.5 c.c. of water at the temperature of the body, leaving no insoluble vehicle or sediment behind.

(13) One-twentieth of it should suffice to kill every sperm in 5 c.c. of human semen at the temperature of the body under laboratory conditions in half an hour.

(14) Its spermicidal power should not be affected by acidity or alkalinity within the range to which it is likely to be subjected.

(15) It should contain no substance which could induce any genetic modification in those sperms which were not killed outright by it. This is a subject upon which nothing is known, beyond the fact that there are no statistics showing an increase in the birth-rate of abnormal individuals since the use of chemical contraceptives became widespread.

No pessary on the market approaches this ideal. Douches and pastes may be used by those who have no aesthetic objection to the use of special appliances for insertion, though douches must be less certain in action from being used after coition.

#### METHODS OF RESEARCH IN CHEMICAL CONTRACEPTION.

It cannot be denied that research in chemical contraception has been haphazard and almost entirely chemical and clinical. The chemical manufacturers seem to have assumed that substances which are known to be good germicides must necessarily be good spermicides. This research has shown this assumption to be erroneous. It suffices to mention the high spermicidal and low germicidal power of sodium oleate.

It seems to me that the subject should be treated in an orderly way, with co-operation between physiologist, chemist, histologist, and clinical worker. Research could be carried out as follows:

(1) The physiologist should test substances for spermicidal powers with guinea-pig sperms.

(2) The chemist should suggest substances for use as vehicles, foam-producers, foam-stiffeners, and reducers of surface tension.

(3) The histologist should test the substances recommended by the physiologist and chemist for effects upon vagina and uterus.

(4) The chemist should decide in what ways the substances passed by the histologist as harmless may be mixed together to form pessaries, in such a way that no substance interferes with the action of another substance.

(5) The physiologist should test the pessary for spermicidal powers with human sperms. I have elaborated a complicated technique for this purpose, which will be described in detail in the next paper of this series.

(6) Those pessaries which are passed by the physiologist as efficient should be tested on women in a birth-control clinic.

### SUMMARY.

1. A technique for comparing the spermicidal powers of pure substances is described in detail. It is hoped that this may be accepted as the standard technique for the purpose.

2. The killing concentration of each substance is determined by this technique. The killing concentration is defined as the lowest concentration, in the series 2, 1,  $\frac{1}{2}$ ,  $\frac{1}{4}$  per cent., etc., which suffices to kill every guinea-pig sperm suspended in glucose-saline solution in half an hour at the temperature of the body in four consecutive experiments, the majority of the control sperms being moderately or very active.

3. 36 substances have been graded by this technique.

4. Mercuric chloride and formaldehyde were found to be the most spermicidal substances. The killing concentration of each is  $\frac{1}{256}$  per cent.

5. Hexyl resorcin kills at  $\frac{1}{64}$  per cent., soaps at  $\frac{1}{32}$  per cent.

6. Formaldehyde and hexyl resorcin, among other substances, seem likely to be useful as contraceptives.

7. The significance of the high spermicidal power of soaps is discussed.

8. Quinine bisulphate and chinisol, which are perhaps more commonly used as contraceptives than any other substances, only kill at  $\frac{1}{2}$  per cent.

9. Certain very poisonous substances have very slight spermicidal powers. This applies to potassium cyanide, prussic acid and strychnine hydrochloride.

10. Foaming mixtures, consisting of acids and sodium bicarbonate, could probably be used alone as contraceptives.

11. The acrosome is the part of the sperm most vulnerable to spermicides. It tends to swell up and burst.

12. Sperms are very susceptible to changes in osmotic pressure.

13. It is the hydrogen ions and not the anions of acids that kill sperms.

14. The suggestion that tribasic acids would be found to be more spermicidal than dibasic, and dibasic than monobasic, is not substantiated.

15. Substances which reduce surface tension are often effective spermicides.

16. The characters of the ideal chemical contraceptive are discussed.

17. The need for co-operation in research in chemical contraception is stressed.

## REFERENCES.

- BAKER, J. R. (1930). The spermicidal powers of chemical contraceptives. I. Introduction, and experiments on guinea-pig sperms. *J. Hygiene*, **29**, 323-9.
- (1930*a*). A fluid for Mammalian sperm-suspensions. *Quart. J. Exper. Physiol.* **20**, 67-70.
- MARTINDALE, W. H. (1928). *Extra Pharmacopoeia*. London.
- MEVES, F. (1899). Über Struktur und Histogenese der Samenfäden des Meerschweinchens. *Arch. f. mikr. Anat.* **54**.
- STOPES, M. C. (1925). *Contraception*. London: John Bale, Sons and Danielsson, Ltd.

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# THE CLINICAL VALUE OF THE COMBINED KAHN AND WASSERMANN TESTS IN THE TROPICS, WITH SPECIAL REFERENCE TO YAWS AND SYPHILIS.

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## INTRODUCTION.

IN tropical Africa the serological diagnosis of yaws and syphilis by means of the Wassermann reaction is notoriously beset with difficulties. The reason for these difficulties is within easy reach. Bigger (1921, 1921 *a*) pointed out that attempts to standardise the Wassermann reaction could never be completely successful, owing to the fact that each of the four reagents employed in the test is more or less variable. This variability becomes far more apparent when the test is undertaken in the tropics. The temperature affects adversely the stability of the reagents, and the precarious conditions attached to the keeping of stock animals interferes with the supply of constantly reliable sera.

A succession of failures directly attributable to local climatic conditions caused me to consider the choice of another test, less sensitive to the vicissitudes of a tropical climate, which would fulfil the requirements of the Wassermann reaction whilst still preserving its diagnostic reliability. The less complex the test and the fewer its reagents, the less liable is it to be affected by conditions of climate and temperature. The ideal test, therefore, would require to combine a minimum of complexity with a maximum of accuracy. At the time the number of tests claiming to equal the Wassermann reaction in value was large and steadily increasing. The Kahn, Sigma, Sachs-Georgi, Meinicke, Müller, Murata, Vernes tests—and these were not all—each had their advocates. Out of these collective methods it was difficult to make a choice, but the first-named appeared to have more supporters than any of the other flocculation tests.

Numerous observers in different countries have at various times reported on the use of the Kahn test performed in parallel with a complement fixation test. McLean (1927) testing 767 sera recorded a 57.6 per cent. agreement between the Kahn and Wassermann tests. Figueira and Trincão (1929) with 1076 sera showed an agreement of 88.1 per cent. and whilst considering the Kahn a more



sensitive test in treated cases, did not find it as diagnostic as the Wassermann. Davenport (1930) carried out 2070 tests by the Kahn technique, and states that in florid cases of secondary syphilis it gave 100 per cent. strongly positive results. He did not find, however, that the changes in treated cases ran parallel with the degree of treatment. Whilst attributing a high degree of sensitiveness and specificity to the test, he believes it to be most useful as a diagnostic procedure rather than a measure of therapeutic response. McIntyre and Gilman (1929) were unable to find sufficient evidence to warrant the supplanting of one test by the other, when comparing the Kahn and Kolmer techniques. They maintain that the clinician and his patient are best served by the performance of both tests. Cookson and Brown (1930) comparing 1000 sera by the Wassermann-Kahn combination found the Wassermann more sensitive even in treated cases, and believe that the Kahn should not supplant it, but that it is most valuable to do both tests. They stressed the simplicity of the Kahn where the Wassermann was not possible, as in the tropics.

Tropical workers have also reported on the Kahn and Wassermann in parallel tests. Butler (1923) with 192 sera and an agreement of 81 per cent., found the Kahn test more sensitive to treatment and apparently more sensitive to yaws than to syphilis. Later (1924) he confirmed his views on sensitiveness with 486 sera. This work was carried out in Tanganyika Territory. Kauntze (1926) in Kenya Colony did not find the Kahn test reliable, except as a rapid method. Pineda (1926) in the Philippines obtained a 89.4 per cent. agreement in yaws cases. Greval (1929) in India, with 1000 of his cases, reported a 69 per cent. agreement between Kahn and Wassermann, and expressed the view that the Kahn test was neither as specific nor as sensitive as the Wassermann. The present writer (1928), in Tanganyika Territory, performed parallel Kahn and Wassermann tests on 86 sera and found an 82 per cent. agreement. Later (1929) with 332 sera he obtained an agreement of 82 per cent.

Reviewing the results of these different observers it is apparent that there is a considerable diversity of opinion regarding the various features of the Kahn test in its relation to the Wassermann reaction. Not only have discrepancies been recorded regarding its actual agreement with the Wassermann test, but opinions have been divided concerning its specificity, sensitiveness and relation to treatment. It will be observed that none of the tropical workers previously mentioned have attributed their inconclusive results directly to the influence of climate: indeed, such influences would be more likely to affect the Wassermann itself rather than the less complex Kahn reaction.

The explanation of anomalous results may be found in several factors other than those associated with climatic conditions. Of these, the personal element is an important feature. There is little doubt that flocculation tests, although credited with a reputation for simplicity, have earned that reputation more in their theory than in their practice. Flocculation tests are admittedly less complicated than complement-fixation tests, because of the smaller number of variable reagents used: but their technique does not because of that require

any less accuracy or skill in its performance. Another factor which may throw some light on the lack of correlation between the results of different workers is the nature of the nomenclature ordinarily employed in the Kahn method. Fine degrees of strong and weak positive reactions must be recorded in the purely scientific research of specificity and sensitiveness: but in general use a nomenclature involving the use of six different signs is more likely to produce discrepant results than one which is confined to three. For the purposes of clinical comparison, the simple "positive," "doubtful" and "negative" notation seems to be of more practical value. Finally, most comparisons regarding the utility of any test have been made in terms of the agreement existing between that test and another serological standard. In the cases under review, that standard has been the Wassermann reaction or one of its modifications.

Davenport (1930) states that "the true value of any test may best be established by comparison with the clinical observation." In the opinion of the writer, most value should be obtained in the use of two well-established tests interpreted in relation to each other and to the clinical data. This procedure should prove particularly useful in the tropics. Here, multiple diseases are frequently present in natives, and may in some way alter their serological state, so that the interpretation of the tests must be made with due regard to the combined clinical and serological observations.

In May, 1928, the League of Nations Health Organisation held a Laboratory Conference on the Serodiagnosis of Syphilis at Copenhagen. In an attempt to establish some standardisation of serodiagnostics methods, they invited distinguished serologists from different parts of the world to perform their various tests on a common batch of sera. From that conference a mass of instructive information has been derived. Full details of the findings may be obtained by referring to the League's *Report*. It might not be out of place, however, to touch briefly on the principal points arising out of the conference:

(1) Of the flocculation tests, the Kahn showed no non-specific reactions, and with the Müller test, gave considerably more positive reactions with syphilis cases—303 out of 495—than any other testing this number.

(2) The highest number of positive Bordet-Wassermann reactions in 495 syphilis cases showing no non-specific reaction was 208.

(3) The Kahn and Müller tests proved more delicate than any other.

(As Kahn has pointed out (1929) the Müller test lacked practicability, whilst the Kahn test fulfilled the four requirements of the conference—practicability, specificity, sensitiveness and clear-cut reactions.)

The conference suggested the following main resolutions:

(a) The best of the flocculation tests is equal in value to the best of the Bordet-Wassermann, but flocculation tests despite their apparent simplicity are very sensitive to differences in experimental conditions and must be in the hands of well trained serologists.

(b) The most reliable information for the clinician is derived from at least two methods.

(c) Serologists should check the accuracy of their results by frequent reference to clinical data.

(d) An uniform notation is desirable, that suggested being + to indicate results which in the view of the pathologist are given only by sera from cases of syphilis (and a few well-defined conditions);  $\pm$  to denote reactions which are neither negative nor positive; — to denote negative reactions.

(e) In the absence of a clear history or signs of syphilis, a positive reaction should not be accepted until a test of at least one more specimen has afforded the same result.

(f) The value of reports on tests of serum would be enhanced if clinicians would study closely the diagnostic and therapeutic implications of such reports.

Other suggestions were made which need not be mentioned here.

It will be observed that most of the causes of confusion existing in the results of different workers were touched upon in the resolutions of the conference, and that methods of avoiding such confusion were recommended.

#### THE AUTHOR'S OBSERVATIONS.

The present work has been carried out, and the results thereof interpreted in accordance with the resolutions put forward by the conference. The writer has not discovered in the literature any detailed record of a combination of the Kahn and Wassermann reactions performed in East Africa under these conditions and it is hoped that some account of the work may be of general interest.

The purposes of the investigation were the following:

(1) To examine the practicability of the Kahn test as a serodiagnostic method in the tropics.

(2) To compare its results with the results of the Wassermann reaction carried out in parallel and under similar conditions.

(3) To assess the value of the tests in combination and singly in relation to the clinical data.

The question as to whether either test was capable of demonstrating a serological difference between yaws and syphilis could not be undertaken satisfactorily. Most of the sera were from yaws cases, and in many instances no detailed information was received regarding the clinical types.

The Wassermann reaction employed was the No. IV method recommended by the Medical Research Committee, and differed only from the original in that it was carried out volumetrically with capillary pipettes.

The "Routine Diagnostic Test" described by Kahn (1928) was used, and his original description was closely followed.

In recording the first series of these tests (1929), the writer made use of Kahn's notation and the Medical Research Council's notation in the respective tests. Since the publication of the League of Nations' resolutions, the total number of tests has been re-interpreted by the nomenclature recommended by

the conference. This has served to alter materially the percentage of agreement between the two tests.

The notation used in the writer's previous reports on 332 tests was:

Kahn		Wassermann
+ + + +		
+ + +	corresponding to	+ +
+ +	" "	"
+ and ±	" "	±
-	" "	-

In the present report the results have been interpreted in both tests as +, ± and -. This correlates with the previous notation thus:

League of Nations	Kahn	Wassermann
+	+ + + +	+ +
	+ + +	+
	+ +	
±	+ or ±	±
-	-	-

500 sera were examined in parallel. The total results were as follows:

Kahn	Wassermann		
	+	±	-
+	191	187	3
±	34	4	16
-	275	1	2
		272	
	500	192	21
			287

The actual correlation between the two tests was therefore 95 per cent. Of the 5 per cent. disagreements, 80 per cent. were due to the Kahn giving more positive and doubtful results than the Wassermann.

230 of the sera were from patients diagnosed as suffering from yaws and syphilis, 218 were from patients diagnosed as not suffering from either of these conditions, and of the remaining 52 sera no details were received.

As these investigations are primarily concerned with the clinical value of the tests, these 52 sera from unknown sources will, for the purposes of the investigations, be discarded. All the results discussed in the remainder of this report will therefore be regarded as applying to the 448 diagnosed cases only.

The total results from the clinical standpoint were:

Kahn	Wassermann		
	+	±	-
+	182	179	3
±	25	1	14
-	241	1	1
		239	
	448	181	18
			249

The correlation is 96.4 per cent. The positive, doubtful and negative results by the tests singly and in combination is shown in the following table:

	+	±	-
Kahn and Wassermann reaction agreement	179	14	239
Kahn only ... ..	182	25	241
Wassermann reaction ... ..	181	18	249

More interest is perhaps attached to the results when stated in relation to the separate clinical diagnoses. In the following tables, each condition is considered separately:

*184 sera from untreated cases of yaws and syphilis.*

Kahn		Wassermann		
		+	±	-
+	156	154	2	0
±	14	1	5	8
-	14	0	0	14
<hr/>		<hr/>		
184		155	7	22

The correlation is 94 per cent. The results by each method are stated below;

	+	±	-
Kahn and Wassermann reaction agreement	154	5	14
Kahn only ... ..	156	14	14
Wassermann reaction only ... ..	155	7	22

The Kahn showed the larger number of positives and doubtful reactions.

*46 sera from cases of yaws and syphilis undergoing treatment.*

Kahn		Wassermann		
		+	±	-
+	17	16	1	0
±	10	0	9	1
-	19	0	0	19
<hr/>		<hr/>		
46		16	10	20

The correlation is 95.6 per cent. The following are the results by each method:

	+	±	-
Kahn and Wassermann reaction agreement	16	9	19
Kahn only ... ..	17	10	19
Wassermann reaction only ... ..	16	10	20

The Kahn gave slightly more positive reactions. The nature and amount of treatment given was not always specified, so that no figures can be given to illustrate the serological changes brought about by any individual forms of treatment.

*218 sera from cases diagnosed as neither yaws nor syphilis.*

Kahn		Wassermann		
		+	±	-
+	9	9	0	0
±	1	0	0	1
-	208	1	1	206
<hr/>		<hr/>		
218		10	1	207

The correlation is 98.6 per cent. The individual findings are:

	+	±	-
Kahn and Wassermann reaction agreement	9	0	206
Kahn only ... ..	9	1	208
Wassermann reaction only ... ..	10	1	207

The Kahn gave more negative reactions.

## SUMMARY OF RESULTS.

(A)	Yaws and syphilis untreated...	...	184 sera
(B)	Yaws and syphilis treated ...	...	46 "
(C)	Neither yaws nor syphilis ...	...	218 "

	Positive			Doubtful			Negative		
	Wasser- mann- Kahn	Kahn	Wasser- mann	Wasser- mann- Kahn	Kahn	Wasser- mann	Wasser- mann- Kahn	Kahn	Wasser- mann
(A)	154	156	155	5	14	7	14	14	22
(B)	16	17	16	9	10	10	19	19	20
(C)	9	9	10	0	1	1	206	208	207
	179	182	181	14	25	18	239	241	249

*Results as percentages of the number of sera in each group.*

(A)	83.1	84.7	84.2	2.7	7.6	3.8	7.6	7.6	11.9
(B)	34.7	36.9	34.7	19.5	21.7	21.7	41.3	41.3	43.4
(C)	4.1	4.1	4.5	0.0	0.4	0.4	94.4	95.4	94.9

Omitting the doubtful reactions, the results may be stated thus:

		% positive	% negative
(A)	Wasserman reaction and Kahn combined	83.1	7.6
	Kahn only ... ..	84.7	7.6
	Wassermann only ... ..	84.2	11.9
(B)	Wassermann reaction and Kahn combined	34.7	41.3
	Kahn only ... ..	36.9	41.3
	Wassermann only ... ..	34.7	43.4
(C)	Wassermann reaction and Kahn combined	4.1	94.4
	Kahn only ... ..	4.1	95.4
	Wassermann only ... ..	4.5	94.9

It will be observed that over 4 per cent. of sera from patients believed not to be suffering from yaws or syphilis gave positive reactions. Strictly, these should be regarded as false positives. Such would be the interpretation of these results if encountered during the course of an investigation of this nature in European practice, where non-syphilitic sera could be obtained with ease. The sera in this series were often sent in the course of ordinary routine and accompanied by a provisional diagnosis. The need of two tests in the tropics becomes more apparent when one realises that doubtful diagnoses are necessarily far from uncommon. Those who are familiar with medical practice in tropical Africa are aware of the difficulty which frequently arises in eliminating completely all suspicion of past or present syphilis and yaws in native patients. It is significant that in the nine cases under review a strongly positive reaction was actually given by both the Khan and the Wassermann tests. One would hesitate before indicting either test on a charge of non-specificity in the circumstances. If it is accepted that the diagnosis in these four cases was incorrect, then one may claim that the Kahn test gave no non-specific reaction. One positive reaction was given by the Wassermann alone on a serum believed not to be from a yaws or syphilis case.

The disagreements may be conveniently analysed together as follows:

	Kahn + Wasser- mann ±	Kahn + Wasser- mann -	Kahn ± Wasser- mann +	Kahn ± Wasser- mann -	Kahn - Wasser- mann +	Kahn - Wasser- mann ±
Yaws and syphilis (untreated)	2	0	1	8	0	0
Yaws and syphilis (treated)	1	0	0	1	0	0
Neither yaws nor syphilis	0	0	0	1	1	1
	3	0	1	10	1	1

The actual cases whose sera did not agree are of interest and are tabulated, together with their clinical and serological diagnoses in the following list:

*Untreated cases.*

	Kahn	Wassermann
(1) Primary syphilis—10 weeks ...	+	±
(2) Friedreich's ataxia... ..	+	±
(3) Secondary yaws ... ..	±	±
(4) Tertiary syphilis ... ..	±	-
(5) Yaws ... ..	±	-
(6) Primary syphilis ... ..	±	-
(7) Secondary syphilis... ..	±	-
(8) Crab yaws ... ..	±	-
(9) Seedy yaws... ..	±	-
(10) Crab yaws ... ..	±	-
(11) Tabes dorsalis ... ..	±	-

*Treated cases.*

(12) Gangosa—history of yaws ...	+	±
(13) Syphilis—previously + both tests...	±	-

*Not yaws or syphilis.*

(14) Leprosy ... ..	±	-
(15) Not diagnosed ... ..	-	+
(16) Hemiplegia—no history of syphilis	-	±

Thus it will be seen that over 80 per cent. of errors in agreement were due to the larger number of positive and doubtful reactions given by the Kahn test. Over 60 per cent. were attributable to ± reactions given by the same test in yaws and syphilis cases. The three Kahn + tests were all accompanied by a ± Wassermann reaction, and it is of interest to note that the clinical condition in each case was one in which a doubtful reaction would commonly be seen.

Kahn claims for his technique a sensitiveness to the effects of treatment which is not observed in other tests. This is due to the three-tube technique, and was actually observed frequently during the present work. The nomenclature adopted tends to mask this phenomenon.

It may be said from analysis of all these results that, in the series of tests under review, the Kahn test satisfied at least three of the four requirements demanded by the League of Nations' Conference—namely, practicability, sensitiveness and specificity. The fourth requirement—clear-cut reactions—did not, in my experience, appear to be satisfied as fully by the Kahn as by the

Wassermann test. This is no doubt a personal view which may alter with increasing practice.

The Kahn test proved its practicability for use in the tropics by the fact that its only special reagent—cholesterinised antigen—showed itself to be as stable as the Wassermann reagents, even when kept for two months at room temperature. Sensitiveness was shown by the large number of its positive reactions which agreed with the clinical findings. Specificity was manifest by the absence of any undoubted false positive result. If the results of the test are interpreted in strict accordance with the League of Nations' resolutions it should prove a reliable diagnostic agent, and even a measure of the progress of treatment, in the tropics and where the Wassermann test is not practicable. When both tests are available, however, it is most instructive to perform them in parallel, the one checking rather than supplanting, the other.

#### SUMMARY.

1. Sera from 500 patients, mostly natives of East Africa, were tested by the Kahn and Wassermann tests, under conditions described.
2. Clinical data were available in the case of 448 specimens. The results of tests upon 230 sera from cases believed to be yaws and syphilis and 218 cases believed not to be yaws and syphilis are discussed. In 46 of the 230 cases, treatment had previously been given.
3. In interpreting results, the resolutions of the League of Nations' Conference on the Serodiagnosis of Syphilis were strictly followed.
4. In the performance of the tests recognised standard methods were used.
5. The two tests correlated exactly in 96·4 per cent. of all cases.
6. The Kahn test was the more sensitive to yaws and syphilis.
7. The Wassermann test alone gave one non-specific reaction.
8. The reagents used in the Kahn test did not deteriorate when kept for two months at room temperature.
9. It is suggested that although the use of both tests is preferable when practicable, the Kahn test alone is a reliable diagnostic measure in the tropics.

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## REFERENCES.

- BIGGER, J. W. (1921). The standardisation of suspensions of red blood cells for Wassermann tests. *Lancet*, ii, 1369.
- (1921 a). The reliability of the Wassermann reaction as performed by different pathologists. *J. Hygiene*, 20, 383-9.
- BURKE-GAFFNEY, H. J. O'D. (1928). *Tanganyika Territory. Annual Report of the Medical Laboratory, Dar-es-Salaam for the Year 1928*, p. 31. H.M. Stationery Office.
- (1929). *Tanganyika Territory. Annual Report of the Deputy Director of Laboratory Service for the Year 1929*. (In the press.)
- BUTLER, G. G. (1923). *Tanganyika Territory. Annual Medical and Sanitation Report for 1923; Report of the Laboratory, Dar-es-Salaam*. H.M. Stationery Office.
- (1924). *Ibid.* 1924.
- COOKSON, H. A. and BROWN, K. S. (1930). Comparison of 1000 serums tested by the Kahn and Wassermann methods. *Brit. Med. J.* i, 441.
- DAVENPORT, K. M. (1930). The clinical value of the Kahn test. *J. Amer. Med. Ass.* 94, 1736-8.
- FIGUEIRA, L. and TRINCAO, C. (1929). Le sérodiagnostic de la syphilis par les réactions de Kahn et de Wassermann. *C.R. Soc. de Biol.* 1, 423-5.
- GREVAL, S. D. S. (1929). The Kahn, Microkahn and Wassermann tests. A comparison in 2066 cases. *Ind. J. Med. Res.* 16, 1009-18.
- KAHN, R. L. (1925). *Serum Diagnosis of Syphilis by Precipitation*. Baltimore: Williams and Wilkins, Co.
- (1928). *The Kahn Test*. London: Baillière, Tindall and Cox.
- (1929). The League of Nations' Conference on laboratory test for syphilis. *J. Amer. Med. Ass.* 93, 351-3.
- KAUNTZE, W. H. (1926). *Colony and Protectorate of Kenya. Annual Report of the Medical Research Laboratory for the Year 1926*.
- LEAGUE OF NATIONS (1928). Health Organisation. Report of the 2nd Laboratory Conference in the Serodiagnosis of Syphilis, held at Copenhagen, May 21st-June 4th, 1928. *Bull. Hyg.* 5, 82-3 (reviewed).
- MCINTYRE, M. C. and GILMAN, R. L. (1929). A clinical comparison of the Kolmer complement fixation and the Kahn precipitation tests in syphilitic and non-syphilitic cases. *J. Amer. Med. Ass.* 93, 358-60.
- MCLEAN, D. (1927). Observations on the relative value of the Wassermann and Kahn tests in cases of congenital syphilis. *J. Path. and Bact.* 30, 182-4. (Abstracted.)
- MEDICAL RESEARCH COMMITTEE (1921). *Special Report Series No. 14. The Wassermann Test*. H.M. Stationery Office, 1918. Reprinted 1921.
- PINEDA, E. V. and WADE, H. W. (1926). The public health value of the Kahn precipitation test, with special reference to yaws. *Trop. Dis. Bull.* 33, 929. (Abstracted.)
- WYLER, E. J. (1929). The Wassermann Test: Technical details of No. 1 method, M.R.C. (modified). *Medical Research Council. Special Report Series, No. 129*. H.M. Stationery Office.

# SEROLOGICAL VARIETIES OF TYPHUS FEVER.

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## I. INTRODUCTION.

To most bacteriologists the serology of typhus fever ceased to be an interesting problem when the diagnostic significance of the agglutination reaction with *B. proteus* X 19 had been generally recognised and the insignificance of this organism as the aetiological factor in typhus fever had been generally proclaimed. In recent years, however, a fresh interest has been aroused in view of the occurrence in endemic form of typhus or typhus-like fevers reported from various parts of the world, where their existence had hitherto not been established.

Fairly comprehensive reviews of these diseases have been published by Burnet and Durand (1929) and by Fletcher (1930). Out of the long list of these typhus-like fevers the following are the best known, since they have been the subject of numerous and valuable investigations: the endemic typhus (Brill's disease) of the United States of America (Maxcy and co-workers, 1923); the endemic typhus of Australia (Hone, 1922); the tropical typhus of the Federated Malay States (Fletcher and co-workers, 1925); the "fièvre exanthématique" of Marseilles (Olmer, 1925, and others).

The clinical symptoms of these diseases differ but little from those seen in mild cases of classical European typhus. Their epidemiology, however, is still obscure; there is no evidence of transmission by lice, no tendency for the disease to spread from man to man and the rôle of certain suspected parasites

is still hypothetical. The laboratory diagnosis is established by the agglutination reaction with *B. proteus* X 19, with the exception of the "fièvre exanthématique" of Marseilles, where this reaction is negative in the majority of cases.

In the endemic typhus of the United States of America and of Australia the agglutination reaction with X 19 occurs with the same frequency and uniformity as in the classical European form. Havens (1927) found the reaction positive in about 95 per cent. of cases in the United States of America, while in the series of cases reported from Australia (Hone, 1923; Bull, 1923; Wheatland, 1926) it was found positive in 100 per cent. In the Federated Malay States, however, Fletcher and his co-workers (1925-30) have established the important fact that the cases of endemic typhus occurring there, and called by them "tropical typhus," belong to two groups, distinctly different in their serum reactions: one group, occurring among the urban population, which reacts with the usual X 19 strains in the same way as cases of typhus fever in other parts of the world; and a second group of cases, occurring among the rural population, whose serum invariably fails to react with the usual X 19 strains, but reacts specifically with one particular strain of X 19, called the "Kingsbury" strain. Fletcher and Lesslar (1926) originally designated the urban group as "Group W" and the rural group as "Group K," according to the designation of the two cultures of *B. proteus* X 19 used in this differential diagnosis; these two cultures were named the "Warsaw" strain and the "Kingsbury" strain respectively. Recently, the name "shop-typhus" has been suggested for the urban (W) group and "scrub-typhus" for the rural (K) group, according to their different epidemiology (Fletcher, 1930).

It is obvious that the facts established by Fletcher and Lesslar are of the greatest importance for the practice of the serological diagnosis of typhus and typhus-like fevers. Moreover, they have a direct bearing on the intricate question of the relationship of *B. proteus* to this group of diseases. The following investigation was, therefore, undertaken with the view of testing the antigenic relationship between the so-called Kingsbury strain of *B. proteus* X 19 and genuine and variant strains of *B. proteus* X, known from the older work of Weil and Felix.

This investigation was made possible by the kind co-operation of Dr Fletcher and Dr Lewthwaite of the Kuala Lumpur Institute, who, during the last two years, supplied us with sera from a considerable number of patients suffering from the two types of tropical typhus and from tsutsugamushi disease occurring in the Federated Malay States. Our thanks are also due to Dr K. F. Maxcy of the United States Public Health Service, Washington; Dr J. Goldberzanka of the State Institute of Public Health, Warsaw; Prof. W. J. Wilson of the Queen's University, Belfast; Dr N. C. R. Keukenschrijver of Sumatra; Prof. R. Kawamura of the Niigata Medical College, Japan; Dr L. B. Bull of the Government Laboratory, Adelaide, Australia. We are glad of this opportunity of thanking all of them for their kindness in sending us sera from patients suffering from typhus and various typhus-like diseases.

## II. THE TWO SEROLOGICAL TYPES OF TROPICAL TYPHUS (FLETCHER AND LESSLAR).

Fletcher and Lesslar (1925, 1926) published numerous and thoroughly observed curves of agglutinin formation in the serum of patients belonging to the two serological groups of tropical typhus. These curves demonstrated convincingly that the relationship between the "Kingsbury" strain and group K sera was as specific as that between the "Warsaw" strain and group W sera. No observation, however, was recorded by these workers to indicate the type of agglutination observed, whether due to O or H agglutinins.

In classical typhus the Weil-Felix reaction is invariably due to O agglutinins in the patient's serum as well as in the serum of animals infected with typhus virus (Weil and Felix, 1917 *b*, 1921). It appeared, therefore, necessary to test sera of the two types of tropical typhus with the view of excluding the possibility of H agglutinins being responsible for the divergence in reaction in the two groups. This seemed to be the more necessary since the cultures used by Fletcher and Lesslar, according to the description given by them, undoubtedly were H variants of *B. proteus* (motile bacilli, spreading film-like growth on agar). The result of this examination is shown in Table I.

Table I. *The two serological groups of tropical typhus in the Federated Malay States.*

	Case No.	Serum examined in London on	Blood drawn in Kuala Lumpur on	Day of illness	Titre of agglutination with strains					
					HX 19	OX 19	HX 2	OX 2	HXK	OKX
Group W = Urban group ("Shop-typhus")	1	3. iv. 29	1. iii. 29	?	2000	5000	0	0	0	0
	2	19. xi. 29	14. v. 29	?	1000	2000	100	100	0	0
	3	19. xi. 29	16. vii. 29	?	1000	2000	100	100	0	0
	4	11. ii. 30	27. xi. 29	?	2000	5000	0	0	0	0
Group K = Rural group ("Scrub-typhus")	1	26. vi. 28	25. v. 28	21	0	0	0	0	10,000	20,000
	2	26. vi. 28	18. v. 28	19	0	0	0	0	5,000	10,000
	3	9. ii. 29	2. i. 29	23	0	0	100	100	2,000	5,000
	4	9. ii. 29	11. vi. 28	22	0	0	0	0	5,000	10,000
	5	9. ii. 29	4. vii. 28	13	0	0	0	0	1,000	2,000
	6	3. iv. 29	?	?	0	0	0	0	1,000	2,000
	7	27. vi. 29	14. v. 29	?	0	0	0	0	1,000	2,000
	8	27. vi. 29	13. v. 29	?	0	0	0	0	500	1,000
	9	11. ii. 30	20. xi. 29	?	0	0	0	0	1,000	2,000
	10	11. ii. 30	20. xi. 29	?	0	0	0	0	2,000	5,000

Agglutination with fresh saline suspensions of living bacteria. Total volume, 1 c.c. Reading after 20 hours, 2 hours' incubation at 37° C., then at room temperature. Titre = highest dilution showing partial agglutination (estimated with the naked eye). Titre 0 = a negative result in dilution 1:100.

Sera from patients in the Federated Malay States, belonging to the two serological types of tropical typhus, have been tested against the H and O variants of the Kingsbury strain and of X 19 and X 2, the two serological types of *B. proteus* X strains known from the work of Weil and Felix. From a comparison of the agglutination titres for the corresponding pairs of O and H variants it is seen that the reactions are invariably due to O agglutinins, since—with the exception of type X 2—the O variants react in every instance up to a higher serum dilution than the corresponding H variants. It is further seen that the specificity of reaction established by Fletcher and Lesslar for

the two groups of tropical typhus is fully confirmed by the results tabulated in Table I.

From these two facts, viz. specificity and O type of the agglutination reaction, the conclusion has to be drawn that the so-called Kingsbury strain of *B. proteus* X plays the same rôle in the serum reactions of the cases in the rural group K as do the usual X 19 strains in the urban group W and in endemic or epidemic typhus in other parts of the world.

### III. THE HISTORY OF THE SO-CALLED K OR KINGSBURY STRAIN OF *B. PROTEUS* X.

So far as the history of this strain could be ascertained it was supplied to the Bland-Sutton Institute in 1921 by the National Collection of Type Cultures as a typical strain of *B. proteus* X 19; and as such it was brought out to the Straits Settlements by Dr A. N. Kingsbury in 1923. This strain was the one employed by Fletcher and Lesslar (1925) in their earlier investigations, which led to the discovery of the typhus nature of the endemic fever of the Malay States now known as tropical typhus. Other strains of *B. proteus* X 19 were then obtained from laboratories in different countries and tested as a routine measure in agglutination tests with the result that the two serological groups K and W have been demonstrated.

Fletcher and Lesslar (1926) compared the Kingsbury strain with eight cultures of X 19, which they obtained from various sources, and found that it did not produce indol and did not ferment saccharose and maltose as all these other X 19 strains did; moreover, it showed certain differences in agglutination and absorption tests with rabbit immune sera, although these reactions proved that all the nine strains were related to one another. On the other hand, when they compared the K strain with fourteen cultures of van Loghem's anindologenes group of *B. proteus*, it was found to differ from them serologically, though it resembled them culturally; moreover, it was the only one of these non-indologenic strains which was agglutinated by the blood of persons suffering from tropical typhus. Fletcher and Lesslar concluded that the Kingsbury strain was neither an ordinary member of the indologenic nor of the non-indologenic group.

Wilson (1927) also compared the Kingsbury strain with other X 19 strains and confirmed the differences in the fermentation of saccharose and maltose and in the formation of indol. As regards serum reactions, however, his conclusion—based on some absorption tests—was, that in sera from typhus patients the agglutinins for "Kingsbury" and the X 19 strains were identical.

Since *B. proteus* is flagellated and possesses the two kinds of antigen known as the O and the H, it is obvious that only the qualitative method of serological analysis is capable of disclosing the true antigenic relationship between members of this group.

#### IV. THE ANTIGENIC RELATIONSHIP BETWEEN THE KINGSBURY STRAIN AND GENUINE STRAINS OF *B. PROTEUS* X.

Two serological types of *B. proteus* X strains have been known since the very beginning of the application of the agglutination reaction in typhus fever. The strains X 1 and X 2 were cultivated first (Weil and Felix, 1916) and were used in routine diagnosis during the epidemic of 1915-16. The agglutination with these strains reached modest titres only, 1:500 exceptionally being exceeded, but the reaction proved to be specific. During the course of that epidemic this organism was isolated a further sixteen times from the blood of typhus patients until, at the end of the epidemic, the nineteenth strain was cultivated, which is known as X 19 (Felix, 1916). The eighteen strains isolated first and named type X 2 were serologically identical, and appeared to differ from the type X 19 in one respect only: the latter reacted with the serum of typhus patients in very much higher dilutions than the former, the difference being ten-fold, hundred-fold and even more, while no difference could be established when the patient's serum was replaced by rabbits' sera homologous to either of these two types.

The true difference between the two types was not revealed until they were separated into their O and H variants (Weil and Felix, 1917 *b*). The O cultures OX 2 and OX 19 differ antigenically from each other not less than do the various types of meningococcus, pneumococcus or dysentery bacilli. Judged from the amount of common group agglutinins the recognised serological types of these latter species show even closer relationships than is the case with the two types of X strains. Rabbits, if intensively immunised with OX 19 or OX 2, either do not develop group agglutinins for the heterologous type at all or form very small quantities only, the difference in the titre being similar to that in the serum of typhus patients. Table II demonstrates the similarity in reaction of a patient's serum and an OX 19 immune rabbit serum.

Table II. *Group agglutinins of O type for X 2 in a patient's serum  
and a rabbit immune serum.*

Serum	Type of agglu- tination	Titre of agglutination with strains			
		HX 19	OX 19	HX 2	OX 2
Typhus patient (18th day of illness) from Adelaide, Australia	0	10,000	20,000	400	400
Rabbit immunised with OX 19 (4 intravenous injections of OX 19 heated for half an hour at 60° C.)	0	5,000	10,000	200	200

Moreover, if the usual technique of absorption tests is applied to such rabbits' sera, it is found that the small amount of group agglutinins is not absorbed or only very incompletely absorbed by the homologous organism, which had given rise to their production. An example is given in Table III.

Table III. *Absorption of group and main agglutinins from a patient's serum and a rabbit immune serum.*

Agglutination with strain	Serum of typhus patient from Australia			Serum of rabbit immunised with OX 19		
	Absorbed in dilution 1:25 with		Unabsorbed	Absorbed in dilution 1:25 with		Unabsorbed
	OX 19	OX 2		OX 19	OX 2	
OX 19	<1000	20,000	20,000	<500	10,000	10,000
OX 2	400	<25	400	200	<25	200

In this respect, too, these O immune sera tally with the sera from typhus patients, but differ from those in the typhoid-paratyphoid group, where the group agglutinins are as readily absorbed by the homologous as by the heterologous organism. The fact that the agglutinins for X 19 and X 2 could not be removed by cross-absorption tests from human typhus sera, where they occur simultaneously, had been commented upon in numerous papers dealing with the theory of the X 19 reaction. Since Weil and Felix showed that rabbit O immune sera behave exactly in the same way, it became clear that the phenomenon is due to the very minute overlapping in O antigen between the two types. The difference in sensitiveness of the two reactions, viz. agglutinin absorption and agglutinin production, is the sole responsible factor.

It appeared, therefore, justifiable to consider X 2 and X 19 as two distinct serological types on the same grounds on which serological types have been recognised in various species. If it were not for the existence of the H antigen which is common to their H variants, the close relationship between these two organisms could not be demonstrated by serum reactions. Weil and Felix designated the *B. proteus* X strains as "specific" in contrast to *B. proteus vulgaris*, because they could not be recovered from sources unrelated to typhus. While varying degrees of community of the H antigen were established between the X strains and *B. proteus vulgaris*, the specific OX 19 and OX 2 antigens could not be found in any instance among the cultures of *B. proteus vulgaris* isolated from various sources, including those which existed in various collections prior to 1918 (Weil and Felix, 1918). On the other hand, the examination of twenty-four genuine strains of type X 2 and of thirty-one strains of type X 19, isolated by these and other workers in various parts of Europe and Asia Minor, showed for each type uniformity of antigenic composition.

The so-called "Kingsbury strain of X 19" was compared with six genuine strains of type X 2 and six genuine strains of type X 19 whose histories are known from the older work of Weil and Felix, Zeiss (1918) and others. Some of these strains were used in the form of their H variant only, while others, including the Kingsbury strain, were available in both the H and the O variants. Agglutination, agglutinin absorption and production of agglutinins in rabbits were applied in numerous experiments. It is not proposed to present

the results obtained in tabular form, since the technique of qualitative analysis of H and O antigens is now sufficiently well known.

It was found that the H antigen of the Kingsbury strain is, in part, identical with that common to all X strains, while its O antigen is completely different from both the OX 2 and OX 19 antigens.

With regard to the H antigen it must be stated that, in previous work on the *proteus* X strains, much less attention has been paid to this component than to the O antigen, since it has been recognised that it is the O antigen which separates the two types of X strains from each other as well as from all the types or races of *B. proteus vulgaris*. Such incomplete analysis as has been recorded suggested that the H antigen is uniform in both types of X strains, while its complexity was clearly disclosed as soon as cultures of *B. proteus vulgaris* were included in this analysis. Evidence of the existence of specific and group components in the H antigen of *B. proteus* strains, comparable to those known in the Salmonella group, has been published by Weil and Felix (1917 a, 1918).

Attention was directed chiefly to the analysis of the O antigen of the Kingsbury strain. In absorption tests with pure O rabbit immune sera the complete difference in the O antigens of each of the types X 19, X 2 and Kingsbury was clearly demonstrated. No trace of group agglutinins of the O type could be detected for the Kingsbury strain in the serum of numerous rabbits immunised with several genuine strains of both types X 19 and X 2 and *vice versa*, while the two types X 2 and X 19 produced in some animals group O agglutinins for each other reaching a titre similar to that demonstrated in Table II. In order to exclude the possibility that a supposed minute amount of group O antigen might be destroyed by prolonged exposure to 100° C. (Schütze, 1930), suspensions of O variants heated for half an hour at 58° C. were used for the immunisation of these rabbits, and suspensions of living bacteria were employed for agglutination and absorption tests. Still the result invariably obtained was that no community of O antigen could be established between the K culture and the two types of X strains.

It has been shown by Wassermann (1903) and Pfeiffer (1904) that various species of animals differ considerably in their antibody response to the injection of the same bacterial antigen. Thus the complex structure of an antigen may not be disclosed unless several species of animals are employed for the production of immune sera. Friedberger, Zorn and Meissner (1922), using chickens for this purpose, were able to demonstrate community in the O antigen of the X strains and the culture of *B. pyocyaneus* Z 1 which Neukirch and Kreuscher (1919) had found to be agglutinated by a certain proportion of cases of typhus fever. We, therefore, applied the same procedure and immunised chickens with the K strain as well as with X 19 and X 2. These sera, however, reacted in the same way as those of the corresponding rabbits which were immunised at the same time, *i.e.* no community of O antigen between the K strain and the two X strains was disclosed, although all the



chicken sera had a high O agglutination titre (1:10,000) for their respective homologous organisms.

From the results summarised above and from what is known from the older work on the antigenic relationship of the two types of X strains to each other and to *B. proteus vulgaris*, two alternatives offered themselves for the classification of the so-called "Kingsbury strain of X 19":

(a) This strain could be considered to be an ordinary *B. proteus vulgaris* belonging to Group III in the scheme of Weil and Felix (1917 a, 1918) and mistakenly labelled as X 19.

(b) It could be regarded as a member of the group of the so-called specific *proteus* X strains, representing a third serological type, whose O antigen differs entirely from those of the two types known hitherto, and does not show even that slight degree of relationship that is indicated by the poorly developed group antigen common to OX 2 and OX 19.

We favoured the second alternative, since it did not appear to us justifiable to neglect the actual history of this strain, and since its remarkably specific relationship to one particular group of typhus cases, established by Fletcher and Lesslar and confirmed by our present investigation, is unparalleled by any facts known from the rather eventful history of serological reactions in typhus fever. We, therefore, considered the Kingsbury strain to be a variant derived from the *B. proteus* X 19 which Dr Kingsbury originally obtained from the National Collection of Type Cultures. To test this hypothesis the Kingsbury strain was compared with variant strains of *B. proteus* X.

#### V. THE ANTIGENIC RELATIONSHIP BETWEEN THE KINGSBURY STRAIN AND VARIANT STRAINS OF *B. PROTEUS* X.

Antigenic variants of *B. proteus* X strains due to alteration of their O antigen have been described by Weil (1920, 1922) and by Felix (1922). Following the procedure of Baerthlein (1918) Weil succeeded in isolating from a single cell culture of OX 19 a variant strain, which antigenically differed completely from the parent strain and showed, by marked group agglutination with OX 2, a closer relationship to this type than to its parent strain. By analogy, X 2 was considered to be a variant of X 19. Felix confirmed and amplified this finding in a study of numerous variants derived from several genuine X strains of both types.

This antigenic variation is concerned with the O antigen only, like Arkwright's (1921) rough variation, with which it is in some way associated. As can be seen from the papers quoted above, numerous variants showing varying degrees of "roughness" have been encountered among the variants of *B. proteus* X; no attention, however, has been paid to the criteria of "roughness" known from the work of Arkwright and others, the sole interest being directed to the serological investigation of the variants.

The definition suggested for this kind of antigenic variation is: that it

leads to the complete loss of the original main O antigen of the parent organism and to the production of another entirely different O substance in the variant. By the isolation of intermediate forms it was shown that the antigenic variants are not formed suddenly but are the result of a gradual change in two directions, viz. the gradual destruction of the original antigenic substance, and the gradual formation of the new substance. The final products of this complex process, the antigenic or serological variants as previously defined, can only be classed as serological types, analogous to those occurring in nature and recognised in many bacterial species. Though the range of this variation is very wide and numerous variants with entirely different O antigens have been derived from the same parent culture as well as from other strains of the same type, still the differentiation between *B. proteus* X and *B. proteus vulgaris* remains definite, and by O agglutination the latter group can be as sharply distinguished from the variants as from the genuine X strains.

Ten of these variants which had survived in agar stabs eight years were available for comparison with the Kingsbury strain. Seven had been derived from four genuine strains of the X 19 type, while three originated from three genuine cultures of the X 2 type. The re-examination of these variants showed that they had maintained their antigenic properties exactly as they had been described originally (Felix, 1922). However, when tested for "roughness" by Arkwright's technique (in saline solutions of increasing salt concentration) varying degrees of roughness were disclosed as is seen from Table IV. This table also shows the reaction of the ten variants tested with pure O sera of rabbits immunised with OX 19, OX 2 and the Kingsbury strain, respectively.

Table IV. *Serum and salt agglutination of variant strains of B. proteus X.*

Agglutination with variant strain	Type of parent strain	Serum agglutination (in normal saline).			Salt agglutination NaCl (%)		
		Dilution 1:200 of O serum of rabbits immunised with strains					
		Kingsbury	X 19	X 2	0.85	1.7	3.4
IR	X 19	±	+++	+++	±	+++	+++
H III	"	-	+++	+++	-	±	+++
OX 19 b 4	"	-	+++	+++	-	++	+++
OS 1, No. 1	"	+++	-	-	-	±	+
S 1, Tr. 2	"	-	-	±	-	-	+
33,180, No. 3	"	±	±	±	±	++	+++
X 21, No. 3	"	+++	-	-	-	±	+
OX 2, No. 4	X 2	-	-	+++	-	±	±
311, No. 3	"	-	-	-	-	-	-
42,428, No. 3	"	-	-	-	-	-	-
Strain		Controls					
Kingsbury		+++	-	-	-	-	-
X 19		-	+++	-	-	-	-
X 2		-	-	+++	-	-	-

The variants are here denoted by the same symbols which were used in the original description (Felix, 1922). Some of them are agglutinated weakly or strongly by the serum corresponding to the type of their parent, while others do not react at all. Some are disclosed as "rough" by normal saline, others

only by salt solutions of higher concentration. The most interesting results, however, are those under the heading of the Kingsbury serum: two out of the ten variants tested are agglutinated by this serum, viz. OS 1, No. 1, and X 21, No. 3.

The former is a descendant of a genuine type X 19 strain, isolated by Zeiss (1918) in Smyrna and designated S 1; the latter is derived from a genuine type X 19 strain isolated by Weil in Poland (1917) and designated X 21.

Since both these variants, because of their agglutinability by 1·7 per cent. salt solution, are characterised as rough, and serum agglutination with suspensions of rough organisms is known to be liable to many pitfalls, some attempts have been made to isolate corresponding smooth variants. These attempts, however, remained unsuccessful. The following detailed analysis of these two variants and the Kingsbury strain was, therefore, conducted in such a way as to employ the variants chiefly for the production and absorption of agglutinins, while agglutination tests were mostly set up against the Kingsbury strain, whose "smoothness" throughout the course of this investigation remained as perfect as that of all the genuine X strains.

Rabbits were immunised by intravenous injections of suspensions of the two variants OS 1, No. 1, and X 21, No. 3, and their parent strains S 1 and X 21. Since the latter were available as H variants only, suspensions heated to 100° C. for 2 hours had to be used in order to obtain pure O sera. Both variants produced in the greater number of rabbits immunised, marked group O agglutinins for the Kingsbury strain, while their respective parent strains entirely failed to do so, like all the other genuine X strains which had already been tested with invariably negative results, as stated in the preceding section.

Table V. *Group O agglutinins for the Kingsbury strain in the serum of rabbits immunised with B. proteus X variants.*

		Serum of rabbits before and after immunisation with suspensions heated at 100° C. of strain												Controls NaCl (%)		
Agglutination with the H form of strain	Serum dilution	Rabbit 265		Rabbit 274		Rabbit 278		Rabbit 269		Rabbit 270						
		Variant OS 1	Variant OS 1	Variant OS 1	Variant OS 1	Variant X 21	Variant X 21	Genuine S 1	Genuine S 1	Genuine X 21	Genuine X 21	0·85	1·7	3·4		
S 1	1:25	-	-	-	-	-	-	+++	-	+++	-	.	.	.		
	1:50	-	-	-	-	-	-	+++	-	+++	-	.	.	.		
	1:100	-	-	-	-	-	-	+++	-	+++	-	.	.	.		
	1:200	-	-	-	-	-	-	+++	-	+++	-	.	.	.		
X 2	1:25	-	-	-	±	-	+±	+++	±	-	-	.	.	.		
	1:50	-	-	-	-	-	±	±	-	-	-	.	.	.		
	1:100	-	-	-	-	-	-	-	-	-	-	.	.	.		
	1:200	-	-	-	-	-	-	-	-	-	-	.	.	.		
Kingsbury	1:25	-	+++	-	+++	-	+++	±	±	-	-	.	.	.		
	1:50	-	++	-	±±	-	±±	-	-	-	-	.	.	.		
	1:100	-	±±	-	±	-	±	-	-	-	-	.	.	.		
	1:200	-	±	-	-	-	-	-	-	-	-	.	.	.		
Titre for homologous strain		.	1:2000	.	1:2000	.	1:5000	.	1:10,000	.	1:5000	.	.	.		

+++ , ++ , + , ± = varying degrees of agglutination, estimated with the naked eye.

The degree of agglutination indicating the "titre" in all the other tables corresponds to the sign +.

Table V illustrates some of the results obtained in these rather numerous experiments. It is seen that the variant sera possess relatively high titre group agglutinins for the strain Kingsbury, whereas they have none for the strain S 1, which is the parent strain of one of these variants. The other parent culture, X 21, was also tested along with the usual X 19 culture; since the results obtained were identical with those for S 1 they are omitted from the table. Neither of the parent strains produced any trace of group agglutinins for the Kingsbury culture (rabbits 269 and 270).

It has been shown in the previous section that the amount of group O antigen common to the two types of *B. proteus* X strains is very much smaller than that known in many other species, particularly among the members of the Salmonella group. If this well-established fact is taken into consideration the results demonstrated in Table V can only be interpreted as indicating definite antigenic relationship between the Kingsbury strain and the two variants. Judged from the incidence and the titre of group O agglutinins produced in the rabbit, this relationship is even closer than that between the types X 2 and X 19, and still more so when the variants are compared with their parent cultures.

The objection might be raised that the "roughness" of the two variants was responsible for the community of O antigen established, since the view is held by some workers that rough variants possess a "cosmopolitan" antigen (Schütze, 1921). This objection, indeed, would carry little weight with the Kingsbury strain, whose perfect smoothness was always thoroughly checked. Nevertheless, the following control was added: rabbits were immunised with the variants H III and 33,180, No. 3, whose roughness is equal to or even greater than that of variants OS 1, No. 1 and X 21, No. 3 (see Table IV) and no trace of group O agglutination with the Kingsbury strain could be detected in these sera.

The relatively high degree of community in O antigen established by the experiments described above suggested the possibility of demonstrating it even by absorption tests. It has been shown in a previous section that this procedure is unsuccessful with the types X 2 and X 19 (see Table III). In the case of the Kingsbury strain and the two variants, however, it proved satisfactory beyond expectation.

Table VI. *Absorption tests with Malayan patients' sera of the K type.*

Serum from typhus patient of the K group	Titre of agglutination with strain Kingsbury. Serum dilution 1:200 absorbed with suspensions of living bacilli of strains								Serum unabsorbed
	Kings- bury	Variant OS1, No. 1	Variant X21, No. 3	S1 genuine	X21 genuine	HX19	OX19	HX2	
Case 1	<200	<1000	<200	10,000	10,000	10,000	10,000	10,000	10,000
Case 2	<200	< 500	<200	5,000	5,000	5,000	5,000	5,000	5,000

In Table VI absorption tests with two sera from Malayan patients suffering from the K type of tropical typhus are reproduced. It is seen that the

Kingsbury agglutinins are absorbed completely or very markedly by the two variants but are left entirely intact by the genuine X cultures, including the two parent strains. Most of the K type sera specified in Table I were tested repeatedly in similar experiments, and other variant and genuine X strains were used as absorbents. Special attention was also paid to controls with the two rough variants H III and 33,180, No. 3, but no interaction with a supposed "cosmopolitan" rough antigen could be traced. The invariable result of these numerous absorption tests was in complete agreement with that obtained in the experiments on the production of group O agglutinins in rabbits, and indicated that the community in O antigen between the Kingsbury strain and the variants OS 1, No. 1, and X 21, No. 3, was indeed one of high degree.

These findings, in our opinion, strongly support the hypothesis put forward in the previous section, according to which the Kingsbury strain is a variant derived from the X 19 culture originally obtained from the National Collection of Type Cultures and represents another type of *B. proteus* X. We, therefore, propose to designate this type as *B. proteus* XK and to use the symbols HXK and OXK for its H and O variants, respectively.

#### VI. CULTURAL AND BIOCHEMICAL PROPERTIES OF *B. PROTEUS* X STRAINS.

Little attention was originally paid to the biochemical properties of *B. proteus* X strains, since it was known already that in the *B. proteus* group (as a whole) these characters possess only a low degree of constancy. So far as they have been tested in this direction the following marked differences have been established among genuine strains of the two types X 2 and X 19: some strains invariably failed to liquefy gelatine or coagulated serum, while others did so constantly; the reaction in litmus-milk varied; on agar plates some strains never showed colonies other than those of the spreading film-like H type, whereas others regularly yielded a certain proportion of non-spreading O type colonies (Felix, 1922). Differences of the magnitude described in the case of the Kingsbury strain (Fletcher and Lesslar, 1926; Wilson, 1927) had, however, not been observed between various genuine strains of *B. proteus* X, while variant strains had not been tested in this respect.

Jötten (1919) and Schaeffer (1919) who first compared the cultural and biochemical properties of *B. proteus* X and *B. proteus vulgaris* found that indologenic strains almost invariably fermented maltose and saccharose, whereas the non-indologenic strains did not. The coincidence of these properties has been confirmed by other workers, although exceptions to this rule have also been reported (Wolff, 1922 a).

Now the objection might be raised that the difference in the ability to produce indol and to ferment maltose and saccharose established between the Kingsbury culture and *B. proteus* X strains is irreconcilable with our conclusion that the former is a variant derived from the latter. However, in the light of the present knowledge of bacterial variability this objection would carry but

little weight. No special effort therefore was made to isolate a non-indologenic variant from an indologenic parent X strain in order to meet such an objection. Wolff (1922 *a*), working with a strain of *B. proteus vulgaris* which produced indol and fermented maltose and saccharose, observed the complete loss of these properties when the strain was re-examined one year later. The strain had only rarely been subcultured during this time and, so far as the inadequate serological tests indicated, no correlated alteration in antigenic structure was established in this variant. Instances of variation in the production of indol and in other forms of decomposition of proteins have been described in various species (for references see Arkwright, 1930).

#### VII. OCCURRENCE OF AGGLUTININS FOR VARIOUS TYPES OF X STRAINS IN CASES OF TYPHUS FEVER FROM VARIOUS PARTS OF THE WORLD.

Since the superiority for diagnostic purposes of the type X 19 over the type X 2 has been recognised, the latter has fallen into disuse and the presence of agglutinins for type X 2 has consequently not been tested in cases of typhus fever occurring during various epidemics or in various parts of the world.

In a series of 225 typhus cases examined by Weil and Felix during the epidemic of 1915-16 in Eastern Galicia, only two cases (less than 1 per cent.) were encountered whose serum was devoid of agglutinins for X 2 throughout the whole course of the disease. Similar results were obtained by other workers during that epidemic (Salubritäts-Kommission, 1916). In the following year, however, in a series of 300 typhus cases from Asia Minor, 22 per cent. of cases were negative to type X 2, while the reaction with X 19 was positive in 100 per cent. of cases (Felix, 1917). Since then no adequate observations have been reported.

When the wide range of antigenic variability of *B. proteus* X was established, it was suggested as possible that this might invalidate the serological diagnosis of typhus cases (Felix, 1922). If the antigen which the typhus virus possesses in common with the X strains were, in the infected human body or in the vector, capable of variation to a similar extent as that established with the X strains *in vitro*, irregularity in the serum reaction of typhus patients had to be expected. In a few tests with some of the variants described and with a small number of patients' sera from Poland no indication of such a complication was found at that time. It now appears, however, to have been clearly demonstrated by the two serological types of tropical typhus described by Fletcher and Lesslar.

We have tested the sera of typhus cases from various parts of the world against the three types, X 19, X 2 and XK. The results are shown in Table VII.

It is seen that, irrespective of the origin of the sera, of the height of the agglutination titre with X 19 and of the presence or absence of group agglutinins for X 2, the reaction to type XK is invariably negative. In this respect these sera tally with the serum from cases of Fletcher and Lesslar's group W

of tropical typhus, and also with the serum of rabbits immunised with types X 19 and X 2 which have been shown to be devoid of group O agglutinins for type XK.

Table VII. *Agglutination of various types of X strains by sera from cases of typhus fever from various localities.*

Typhus patients from	Case No.	Serum examined in London on	Blood drawn on	Titre of agglutination with strains					
				HX 19	OX 19	HX 2	OX 2	HXK	OKX
United States of America	1	3. v. 28	10. iii. 27	200	500	0	0	0	0
	2	"	8. vi. 27	500	1,000	0	0	0	0
	3	"	12. xi. 27	200	500	0	0	0	0
	4	3. iii. 30	10. iv. 29	2,000	5,000	0	0	0	0
	5	"	23. v. 29	1,000	2,000	0	0	0	0
	6	"	10. vi. 29	500	1,000	0	0	0	0
	7	"	20. vii. 29	2,000	5,000	0	0	0	0
Poland	1	25. iii. 30	6. iii. 30	2,000	5,000	200	200	0	0
	2	"	"	2,000	5,000	100	100	0	0
	3	"	13. iii. 30	200	500	100	100	0	0
	4	28. v. 30	25. iii. 30	5,000	10,000	500	500	0	0
	5	"	26. iii. 30	2,000	5,000	0	0	0	0
	6	"	1. iv. 30	200	500	0	0	0	0
	7	"	4. iv. 30	500	1,000	0	0	0	0
	8	"	5. iv. 30	1,000	2,000	100	100	0	0
	9	"	10. iv. 30	1,000	2,000	100	100	0	0
	10	"	11. iv. 30	2,000	5,000	100	100	0	0
Ireland	1	12. i. 29	?	2,000	5,000	0	0	0	0
Australia	1	12. i. 29	2. viii. 28	10,000	20,000	400	400	0	0

With regard to the occurrence of group O agglutinins for type X 2, there appears to exist a difference between typhus cases from the United States of America and those of other origin. Most of the sera of typhus cases from Poland specified in Table VII contain significant group agglutinins for X 2, indicating that the agglutinogenic properties of the typhus virus in Poland have remained much the same as they were in 1915-16. Sera from group W cases from the Federated Malay States (see Table I) and the one serum from Australia which we have had an opportunity to examine, react like the sera from Poland. The American sera, however, do not contain any group agglutinins for X 2, and a similar result has been published recently by Spencer and Maxcy (1930). Should this finding be confirmed by investigations on a larger scale, another peculiarity of the virus of endemic typhus of the United States would thus have been established, in addition to the property of producing conspicuous scrotal and testicular lesions in infected animals, which this virus (Maxcy, 1929 *a, b, c*; Pinkerton, 1929) shares with those of the Mexican Tabardillo (Neill, 1917; Mooser, 1928, 1929) and of Rocky Mountain spotted fever.

Two details of a more technical nature must be mentioned in passing. O agglutination generally gives a distinctly higher titre with the O variant than with the corresponding H variant. This is the rule in *B. proteus* (Weil and Felix, 1917 *b*) as well as in the typhoid-paratyphoid group of organisms (Felix, 1930). With the variants of X 19 and XK this was consistently the

case throughout the present investigation. The culture OX 2, however, only yielded titres equal to or even lower than those for HX 2. The reason for this anomaly has not been ascertained. The other anomaly noted was that suspensions of both HX 2 and OX 2 were agglutinated by glycerole highly diluted with normal saline. A number of sera from typhus patients from America could not be included in Table VII, since their agglutination with HX 2 and OX 2 was evidently due to the non-specific action of glycerole which had been used as preservative. All the patients' sera tested in the course of this investigation had been neither inactivated by heat nor preserved by any disinfectant.

#### VIII. H AGGLUTININS AS SOURCE OF ERROR IN THE DIAGNOSIS OF TYPHUS AND TYPHUS-LIKE DISEASES.

The most important source of error in the Weil-Felix reaction is H agglutination with *B. proteus* X strains, since this type of agglutination is of no significance in the diagnosis of typhus (for references see Wolff, 1922*b*). H agglutinins due to an existing or a previous infection with *B. proteus vulgaris* (Groups II and III of Weil and Felix, 1918), such as cystitis, otitis, etc., are occasionally met with in the serum of healthy individuals or of patients suffering from various non-typhus diseases. Unless due attention is paid to the differentiation between H and O agglutinins instances of erroneous typhus diagnosis will necessarily occur and may lead to fallacious conclusions with regard to the occurrence and significance of agglutinins for various types of X strains. The case of "typhus-like" fever recently published from Australia (Penfold and Corkill, 1928) may serve as an example of the misleading effect of this source of error. The serum reactions of this patient are illustrated in Table VIII together with those of two cases from other localities.

Table VIII. *H agglutinins for B. proteus X strains in human sera.*

Case	Patient from	Serum examined in London on	Blood drawn on	Type of agglutination	Titre of agglutination with strains					
					HX 19	OX 19	HX 2	OX 2	HXK	OKK
1	Melbourne, Australia	15. ii. 29	21. iv. 28	O	0	0	0	0	0	0
				H	500	0	500	0	500	0
2	Kuala Lumpur, Federated Malay States	15. ii. 29	3 xi. 28	O	0	0	0	0	0	0
				H	2000	0	2000	0	100	0
3	Warsaw, Poland	25. iii. 30	14. iii. 30	O	2000	5000	0	0	0	0
				H	1000	0	1000	0	200	0

It is seen that the Australian serum, which we received through the kindness of Prof. Wilson, Belfast, contained H agglutinins only, *i.e.* its Weil-Felix reaction was negative. The same was the case with serum No. 2, which was sent to us with the diagnosis of tropical typhus of type W. Serum No. 3, however, from a typhus patient from Poland, gave O agglutination with type X 19, significant for typhus fever, in addition to H agglutination with all three types. It has been mentioned in Section IV that in *B. proteus* the H antigen is of a complex structure and that the Kingsbury strain and types



X 2 and X 19 possess only partly identical H antigens. This finds its expression also in the H titres recorded in Table VIII, which are equally high for all three types in serum No. 1, but differ markedly in the others.

# IX. SERUM REACTIONS IN CASES OF TSUTSUGAMUSHI DISEASE FROM THE EAST INDIES AND JAPAN.

Cases of tsutsugamushi, the endemic typhus-like disease of Japan, are reported to give no reaction with X 19 (Ishiwara and Ogata, 1923; Kawamura, 1926). In the Malayan form of the disease the same result has been obtained with X 19, but with the Kingsbury strain agglutination in low serum dilutions has been observed (Fletcher and Field, 1927; Fletcher, Lesslar and Lewthwaite, 1929). With the kind permission of Dr W. Fletcher the details of his and his co-workers' observations are given in Table IX.

Table IX. *Agglutination of strain Kingsbury in Fletcher and his co-workers' cases of tsutsugamushi in the Malay States. (Personal communication from Dr W. Fletcher.)*

Case no. 1	Day of illness	10	12	14	—	—	—	—	—	—
(European)	Titre for XK	0	80	40	—	—	—	—	—	—
Case no. 2	Day of illness	10	12	22	25	28	35	—	—	—
(European)	Titre for XK	0	60	120	80	60	60	—	—	—
Case no. 3	Day of illness	3	10	15	20	24	34	41	—	—
(European)	Titre for XK	0	10	60	240	240*	100	60	—	—
Case no. 4	Day of illness	6	13	15	18	22	25	30	40	60
(European)	Titre for XK	0	60	60	60	60	60	90	120	30
Case no. 5	Day of illness	10	16	19	23	26	34	—	—	—
(Indian)	Titre for XK	60	240	240	240	120	60	—	—	—
Case no. 6	Day of illness	11	17	—	—	—	—	—	—	—
(European)	Titre for XK	60	60	—	—	—	—	—	—	—
Case no. 7	Day of illness	10	16	19	23	26	34	—	—	—
(Indian)	Titre for XK	160	240	240†	240	120	60	—	—	—

\* Partial 640.

† Partial 480.

Although the titres of the agglutination recorded in these cases are incomparably lower than those known from type K of tropical typhus, still their rise and fall is in several instances regular and distinct. Fletcher and his co-workers did not observe similar agglutination reactions with type XK in control tests with the blood of normal persons or of those suffering from other diseases. They, therefore, ascribed significance to these reactions in spite of the low titres reached and concluded that the two diseases are closely related.

We have tested sera from cases of tsutsugamushi disease from Sumatra and Japan. The results are shown in Table X.

When the results shown in Table X are compared with those recorded in Tables I and VII the conclusion drawn by Fletcher and his co-workers with regard to the relationship between tsutsugamushi and the K type of tropical typhus appears to be well justified. None of the tsutsugamushi sera reacted with the types X 19 or X 2, while the majority gave O agglutination with type XK. The Sumatran sera, Nos. 2 and 10, showing agglutination titres as

high as those met with in tropical typhus of the K type, may possibly belong to cases of this disease which have mistakenly been diagnosed as cases of tsutsugamushi. Both the W and the K type of tropical typhus are reported to occur in Sumatra (Wolff, 1929) and in Java (van Steenis, 1929; Peverelli, 1930) and the clinical differentiation between these diseases and tsutsugamushi is sometimes difficult. Unfortunately, we have been unable to secure the histories of the Sumatran cases.

Table X. *Agglutination of various types of X strains by sera from cases of tsutsugamushi disease.*

Tsutsu- gamushi patients from	Case no.	Serum examined in London on	Blood drawn on	Titre of agglutination with strains					
				HX 19	OX 19	HX 2	OX 2	HXK	OXK
Sumatra	1	29. i. 29	18. ix. 28	0	0	0	0	0	0
	2	"	21. ix. 28	0	0	0	0	1000	2000
	3	"	"	0	0	0	0	200	500
	4	"	24. ix. 28	0	0	0	0	0	0
	5	"	"	0	0	0	0	0	0
	6	"	5. x. 28	0	0	0	0	100	200
	7	"	"	0	0	0	0	100	200
	8	"	9. x. 28	0	0	0	0	0	0
	9	25. vi. 29	23. v. 29	0	0	0	0	0	0
	10	"	"	0	0	0	0	500	1000
Japan	1	11. ii. 30	29. ix. 29	0	0	0	0	100	200
	2	"	30. x. 29	0	0	0	0	100	200
	3	"	28. xi. 29	0	0	0	0	200	500

All the Japanese cases, whose selection we owe to the expert knowledge of Prof. Kawamura (Niigata, Japan) and the Sumatran cases, Nos. 3, 6, 7, tally with regard to their titre for XK with Fletcher's figures recorded in Table IX. The incidence of these agglutinins and their low titre strikingly recall the agglutination of *B. proteus* X 2 with typhus sera from Poland (see Table VII). Unless this analogy is entirely misleading the suggestion appears to be justifiable that the agglutination of type XK in tsutsugamushi is of the same order as that of type X 2 in typhus fever from Poland, *i.e.* group agglutination due to group O antigen which the K type of *B. proteus* X possesses in common with the unknown virus of tsutsugamushi disease. If this hypothesis were proved by the isolation from cases of tsutsugamushi of another serological type of *B. proteus* X corresponding to this virus, the analogy to the history of the X 19 reaction in European typhus would become complete. There, as is known, the group agglutination with type X 2 was found first and led to the discovery of the main agglutination with type X 19.

It appears not unlikely that further serological varieties of typhus may yet be recognised among the different typhus-like diseases in different parts of the world (Megaw, 1921, 1924; Burnet and Durand, 1929; Fletcher, 1930). Recent observations on Rocky Mountain spotted fever and on the "fièvre exanthématique" of Marseilles seem to lend support to this view.

# **X. REVIEW OF DATA PUBLISHED ON SERUM REACTIONS IN ROCKY MOUNTAIN SPOTTED FEVER AND IN THE "FIÈVRE EXANTHÉMATIQUE" OF MARSEILLES.**

We have had no opportunity of testing sera from cases of these two forms of typhus-like diseases. A brief account of the serological data published by various workers may, therefore, serve to complete the survey which we are attempting.

## *(a) Rocky Mountain spotted fever.*

Cases of this disease were first reported to give a negative reaction with X 19 (Kelly, 1923). Kuczynski (1927), however, found that the virus of Rocky Mountain fever produced X 19 agglutinins in infected animals and this result was confirmed by Otto (1928), Munter (1928) and Kerlee and Spencer (1929). The last mentioned workers and Spencer and Maxcy (1930) also established that the reaction was positive in a large proportion of human cases of the disease.

In cross-immunity tests with rats (Kuczynski, 1927) and with rabbits (Munter, 1928), it has been shown that the antigens responsible for the production of X 19 agglutinins are not identical in the viruses of typhus and Rocky Mountain fever, but possess an overlapping component in common. The observations made by the American workers point clearly to the same conclusion.

Kerlee and Spencer (1929) tested a few sera from human cases and found agglutinins for both the X 19 and the XK type together in one serum and separately in others. In their experiments with rabbits the infection with the virus of Rocky Mountain fever led to the production of agglutinins for both types and there was not much difference in the titres recorded for the two strains. Spencer and Maxcy (1930) recently published a comparative study of the agglutination reactions of human cases of Rocky Mountain fever and of endemic typhus of the United States. Sera from forty and sixteen cases of the two diseases, respectively, were tested against the O and H variants of the three types X 19, X 2 and XK. The difference in reaction in the two series was very conspicuous. In endemic typhus it was invariably the type X 19 which reacted with a high titre of agglutination, whereas significant group agglutinins for the two other types were absent. This is in agreement with our own experience (see Table VII). With Rocky Mountain fever, however, there was no uniformity of results: type X 19 reacted significantly in a minority of cases only and the titres of this reaction were markedly lower than those recorded in endemic typhus; moreover, agglutinins for types X 2 and XK were found in a considerable proportion of these cases. Even if allowance be made, in the case of strains HX 2 and OX 2, for the possible non-specific agglutination by glycerole which was probably present in some of the sera used, still the difference in reaction in the two series was striking. Spencer and Maxcy rightly concluded that there was a qualitative difference.

It would appear that two alternatives may be consistent with the established facts, viz. (1) the virus of Rocky Mountain fever may not be uniform

antigenically but may be divided into serological varieties similar to those established by Fletcher and Lesslar in Malayan tropical typhus; (2) the main O antigen of the virus may correspond to a type of *B. proteus* X other than the three types known hitherto, and the irregular reactions which these types give with sera from human cases of the disease may be due to common group O antigens.

(b) "*Fièvre exanthématique*" of *Marseilles*.

Numerous observations on this typhus-like disease have been published since D. Olmer (1925) first called attention to its occurrence in the Marseilles area. The disease is identified by some workers with the "*fièvre boutonneuse*" of Tunis, with the "*febbre eruttiva*" of Italy and with similar fevers occurring in other parts of the Mediterranean (for references see Conseil, 1929). The relationship of this disease to classical typhus is a matter of controversy, chiefly based on the negative results of cross-immunity tests and on the irregular agglutination reactions recorded by various workers. Samples of the same serum when tested in various laboratories against strains of *B. proteus* X have in some instances been recorded as positive by one worker and negative by another.

Burnet and Olmer (1927) published some results of the agglutination reactions obtained in nine cases of the Marseilles fever. Several strains of type X 19 and one of type X 2 were employed and in three out of the nine sera tested the reaction was considered as positive. The titres recorded were low (maximum 1:500) and the agglutination occurred in two cases with both types, and in one case with the type X 19 only. Burnet and Olmer (1927) state that, in an earlier series of forty-two cases observed by Olmer, the Weil-Felix reaction was negative, while Conseil (1929) makes the statement that the reaction was positive in nine out of forty-four of Olmer's cases. Further observations led Olmer (1928) to the conclusion that although some of the cases give a positive Weil-Felix reaction, this reaction is inconstant and quite unlike the results obtained in true typhus. Boinet, Piéri and Dunan (1928 *a, b*) had negative results only with both the X 19 and the XK type.

Assuming that the positive reactions referred to have not been due to some source of error, like H agglutinins in the patient's serum or "roughness" of the cultures used, then the most justifiable interpretation would seem to be that the irregular and low titre reactions with *B. proteus* X strains are group agglutinations due to the agglutinogenic properties of the unknown virus of the "*fièvre exanthématique*." Conseil (1929), however, states that these observations are of no significance since a positive Weil-Felix reaction is often met with in human sera during convalescence after various non-typhus diseases. This unwarranted statement is in sharp contrast to the unanimous opinion of those who have used the test extensively (for references see Otto and Munter, 1930). If the observations published by Burnet and Olmer (1927) were confirmed by adequately controlled tests with H and O variants of

*B. proteus* X they would, in our opinion, strongly suggest that "fièvre exanthématique" is another serological variety of typhus fever.

# XI. THE SERUM REACTIONS OF VARIOUS FORMS OF TYPHUS AND TYPHUS-LIKE DISEASES.

The list of various forms of typhus and typhus-like diseases, whose serum reactions are shown in Table XI, does not include a number of typhus-like fevers reported from various localities but not yet adequately studied. The reactions with *B. proteus* X 19 and XK only are specified in this table because type X 2 has not yet been shown to correspond to the main O antigen of the virus of any serological variety of typhus.

Table XI. *Showing the serum reactions of various forms of typhus and typhus-like diseases.*

Name of disease	Locality	Vector	Agglutination with <i>B. proteus</i> X	
			Type X 19	Type XK
Typhus (epidemic and endemic)	Old and New Worlds	Lice	Positive	Negative
Tabardillo (Mexican typhus)	Mexico	"	"	Not tested
Endemic typhus (Brill's disease)	South Eastern United States	Unknown	"	Negative
Endemic typhus	Australia	"	"	"
"	Rome	"	"	Not tested
Rocky Mountain spotted fever	Western United States	Ticks	Partly positive	Partly positive
Tropical typhus, Type W	Malaya	Unknown	Positive	Negative
" " K	"	"	Negative	Positive
Tsutsugamushi	Malaya and Sumatra	Mites	"	Weakly positive
"	Japan	"	"	"
Typhus-like fever	Central India	Unknown	"	Not tested
Fièvre exanthématique	Marseilles	"	Negative (partly positive)	"

It is obvious that this newer knowledge of serological types in typhus fever has a direct bearing on the practice of serological diagnosis. The evidence of unity among the different forms of typhus hitherto derived from the X 19 reaction is no longer valid. Serological types of the disease may exist whose diagnosis by agglutination will only become possible after the isolation of the corresponding types of *B. proteus* X. Cases of typhus-like diseases as those from the Marseilles district, from Central India (Megaw, Shettle and Roy, 1925), etc. may, therefore, prove to belong to the typhus group, although the X 19 reaction was found negative in these cases.

It is further obvious that the existence of serological varieties of typhus also invalidates the significance hitherto ascribed to negative cross-immunity experiments. Failure to obtain cross-protection between classical typhus and some typhus-like diseases may be due to differences in antigen such as occur in the serological types of bacteria. The typhus nature of a disease which otherwise is typhus-like should, therefore, not be definitely excluded on the basis of negative cross-immunity tests.

## XII. SUMMARY.

1. Fletcher and Lesslar's observations on two serological types of tropical typhus have been fully confirmed.

2. The antigenic relationship between the indologenic *B. proteus* X 19 and the non-indologenic Kingsbury strain is of the same order as that obtaining between the X 19 and X 2 types of *B. proteus* X.

3. The Kingsbury strain is an antigenic variant derived from the original X 19 culture and represents another serological type of *B. proteus* X. The symbol XK is suggested for this type.

4. Sera from cases of classical European typhus and of endemic typhus of the United States of America and of Australia have been tested for the occurrence of main and group O agglutinins for the known types of *B. proteus* X.

5. H agglutination as source of error in the diagnosis of typhus cases is illustrated by some examples.

6. Sera from cases of tsutsugamushi from Sumatra and Japan react with type XK like the Malayan cases of this disease described by Fletcher and co-workers.

7. This latter reaction is of the order of group O agglutination. It is suggested that antigenically the virus of tsutsugamushi corresponds to another serological type of *B. proteus* X which is yet unknown.

8. The data published on the serum reactions in Rocky Mountain spotted fever and in the "fièvre exanthématique" of Marseilles are analysed. It is suggested that these two diseases represent further serological varieties of typhus.

9. The significance hitherto attached to negative agglutination tests with *B. proteus* X and to negative cross-immunity tests obtained with some typhus-like diseases requires revision in the light of recent observations.

## REFERENCES.

- ARKWRIGHT, J. A. (1921). *J. Path. and Bact.* **24**, 36.  
 — (1930). *System of Bacteriology*, Med. Res. Council, London, **1**, 335.  
 BAERTHLEIN, K. (1918). *Zentralbl. f. Bakt. Abt. I, Orig.* **81**, 369.  
 BOINET, PIÉRI, J. and DUNAN, J. (1928 a). *Bull. Acad. Méd.* 3rd Ser. **100**, 949.  
 — — — (1928 b). *Marseille-Médical*, **65**, 703 (Dec. 5).  
 BULL, L. B. (1923). *Med. J. Australia*, **1**, 443.  
 BURNET, E. and DURAND, P. (1929). *Bull. Soc. Path. exotique*, **22**, 85.  
 BURNET, E. and OLMER, D. (1927). *Arch. Inst. Pasteur, Tunis*, **16**, 317.  
 CONSEIL, E. (1929). *Ibid.* **18**, 86.  
 FELIX, A. (1916). *Wien. klin. Wochenschr.* **29**, 873.  
 — (1917). *Zeitschr. f. Immunitätsf.* **26**, 602.  
 — (1922). *Ibid.* **35**, 57.  
 — (1930). *Lancet*, **i**, 505.  
 FLETCHER, W. (1930). *Proc. R. Soc. Med.* **23**, Sect. Epidem. p. 1021.  
 FLETCHER, W. and FIELD, J. W. (1927). *Bull. Inst. Med. Res., Fed. Malay States*, No. 1.  
 FLETCHER, W. and LESSLAR, J. E. (1925). *Ibid.* No. 2.  
 — — (1926). *Ibid.* No. 1.

- FLITCHER, W., LESSLAR, J. E. and LEWTHWAITE, R. (1928). *Trans. Soc. Trop. Med. Hyg.* London, **22**, 161.
- (1929). *Ibid.* **23**, 57.
- FRIEDBERGER, E., ZORN, W. and MEISSNER, G. (1922). *Zeitschr. f. Immunitätsf.* **34**, 259.
- HAVENS, L. C. (1927). *J. Infect. Dis.* **40**, 479.
- HONE, F. S. (1922). *Med. J. Australia*, **i**, 1.
- (1923). *Ibid.* **i**, 435.
- ISHIWARA, K. and OGATA, N. (1923). *Zentralbl. f. Bakt. Abt. I, Orig.* **90**, 164.
- JÖTTEN, K. W. (1919). *Berl. klin. Wochenschr.* p. 270.
- KAWAMURA, R. (1926). *Med. Bull. College of Med., Cincinnati Univ.* **4**, Special Nos. 1 and 2.
- KELLY, F. L. (1923). *J. Infect. Dis.* **32**, 223.
- KHELER, A. L. and SPENCER, R. R. (1929). *Publ. Health Rep., Washington*, **44**, 179.
- KUCZYNSKI, M. H. (1927). *Die Erreger des Fleck- u. Felsenfiebers*, Berlin: J. Springer.
- MAXCY, K. F. (1929 a). *Publ. Health Rep., Washington*, **44**, 589.
- (1929 b). *Ibid.* **44**, 1735.
- (1929 c). *Ibid.* **44**, 1935.
- MAXCY, K. F. and HAVENS, L. C. (1923). *Amer. J. Trop. Med.* **3**, 495.
- MERGAW, J. W. D. (1921). *Ind. Med. Gaz.* **56**, 361.
- (1924). *Ibid.* **59**, 68.
- MERGAW, J. W. D., SHUTTLE, F. B. and ROY, D. N. (1925). *Ibid.* **60**, 53.
- MOOSER, H. (1928). *J. Infect. Dis.* **43**, 261.
- (1929). *Ibid.* **44**, 186.
- MUNTER, H. (1928). *Zeitschr. f. Hyg.* **109**, 124.
- NEILL, M. H. (1917). *Publ. Health Rep. Washington*, **32**, 1105.
- NEUKIRCH, P. and KREUSCHER, A. (1919). *Beitr. zur Klinik d. Infektionskr. u. Immunitätsf.* **8**, 68.
- OLMER, D. (1925). *Marseille-Médical*, Aug. 5 and Nov. 5.
- (1928). *Bull. Acad. Méd.* 3rd Ser. **100**, 996.
- OTTO, R. (1928). *Zentralbl. f. Bakt. Abt. I, Orig.* **106**, 279.
- OTTO, R. and MUNTER, H. (1930). *Handbuch d. pathog. Mikroorg.* hrsg. Kolle, Kraus u. Uhlenhuth, Jena, **8**, 1107.
- PENFOLD, W. J. and CORKILL, A. B. (1928). *Med. J. Australia*, **ii**, 304.
- PEVERELLI, P. (1930). *Geneesk. Tijdschr. v. Nederl. Indië*, **70**, 436.
- PFEIFFER, R. (1904). *Festschr. zu Ehren Robert Kochs*.
- PINKERTON, H. (1929). *J. Infect. Dis.* **44**, 337.
- SALUBRITÄTS-KOMMISSION (1916). *Feldärztl. Blätter, K. u. K. 2. Armee*, No. 11.
- SCHAEFFER, H. (1919). *Zentralbl. f. Bakt. Abt. I, Orig.* **83**, 430.
- SCHÜTZE, H. (1921). *J. Hygiene*, **20**, 330.
- (1930). *Brit. J. Exp. Pathol.* **11**, 34.
- SPENCER, R. R. and MAXCY, K. F. (1930). *Publ. Health Rep. Washington*, **45**, 440.
- VAN STEENIS, P. B. (1929). *Geneesk. Tijdschr. v. Nederl. Indië*, **69**, 572.
- WASSERMANN, A. (1903). *Zeitschr. f. Hyg.* **42**, 262.
- WEIL, E. (1920). *Wien. klin. Wochenschr.* **33**, 61.
- (1922). *Zeitschr. f. Immunitätsf.* **35**, 25.
- WEIL, E. and FELIX, A. (1916). *Wien. klin. Wochenschr.* **29**, 33.
- (1917 a). *Ibid.* **30**, 393.
- (1917 b). *Ibid.* **30**, 1509.
- (1918). *Ibid.* **31**, 637.
- (1921). *Zeitschr. f. Immunitätsf.* **31**, 457.
- WHEATLAND, F. L. (1926). *Med. J. Australia*, **i**, 261.
- WILSON, W. J. (1927). *J. Hygiene*, **26**, 213.
- WOLFF, G. (1922 a). *Zentralbl. f. Bakt. Abt. I, Orig.* **89**, Beih. 225.
- (1922 b). *Ergebn. Hyg. Bakt.* **5**, 532.
- WOLFF, J. W. (1929). *Geneesk. Tijdschr. v. Nederl. Indië*, **69**, 429.
- ZEISS, H. (1918). *Arch. f. Hyg.* **87**, 246.

# THE EXAMINATION OF THE TISSUES AND SOME OBSERVATIONS ON THE BLOOD PLATELETS OF RABBITS AT INTERVALS OF FIVE MINUTES, AND LATER, AFTER INTRAVENOUS INOCULATIONS OF *STAPHYLOCOCCUS AUREUS* AND INDIAN INK.

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(With Plates I-III).

WE have endeavoured in these investigations to obtain a comparison of the immediate reactions in the tissues and blood platelets of rabbits to *Staphylococcus aureus* which is highly pathogenic to these animals, and to suspensions of non-pathogenic particles such as indian ink and colloidal silver, when injected intravenously.

Many workers (Nissen, 1922; Westhues, 1922; Boerner-Patzelt, 1923; Wislocki, 1924; and Lang, 1926) have observed large aggregations of indian ink in the lungs of animals injected intravenously with it.

Cappell (1930) has very carefully studied the fate of colloidal particles injected into the circulation. He found that the injection of indian ink produces at the outset a leucopenia in the peripheral blood, due to an accumulation of polymorph leucocytes in the lung capillaries, some of which take up the particles. The ink, however, is widely circulated, and is found especially in the Kupfer cells in the liver, in the endothelial cells of the splenic sinuses and in the glomerular and intertubular vessels in the kidneys.

Werigo (1894) found in anthracæmia in rabbits that active phagocytosis occurred of *Bacillus anthracis* by the leucocytes in the lungs and the Kupfer cells in the liver. Levaditi (1901) was the first to note that the inoculation of organisms into the circulation results in the accumulation of polymorphs in the lungs. Andrewes (1910) showed that the injection of organisms into the blood stream is followed by an initial leucopenia due to the collection of polymorphs in the lungs, but this occurs only to a slight degree with *S. aureus*.

Bartlett and Ozaki (1917, 1918) found that, in the first 10 minutes after

<sup>1</sup> Working during tenure of a grant given by Mr and Mrs Sannyer Atkin, in memory of their son.



injection of *S. aureus* into the left ventricle, the organism is taken up by polymorphs in the lungs, subsequently by endothelial cells in the liver and spleen, while an increasing number are found free; these eventually lodge in the kidney capillaries, and give rise to abscesses. These authors also state that, as the septicaemia increases, the endothelial cells take up proportionately more and more of the organisms.

Nagao (1920) found that, when he injected formol-killed vaccines of non-haemolytic streptococci intravenously into rabbits, the polymorphs accumulated first and chiefly in the lungs. The extensive researches of Bull (1914-16) led him to the conclusion that non-virulent organisms when injected into the circulation are agglutinated and accumulate in the capillaries of the lungs, liver, spleen and kidneys, where they are taken up by the polymorphs; whereas, if the organisms are virulent for the animal, there is no agglutination and no phagocytosis.

Wright (1927) observed also that it is only in immune rabbits that the agglutination of virulent pneumococci occurs in the lung and liver capillaries with subsequent phagocytosis by the polymorphs and endothelials.

It appears from the literature that the effect of previous immunisation on the rate of clearing of the blood of bacteria varies with each organism. It is stated to be increased in the case of *Vibrio cholerae* (Bail, 1905) and *B. anthracis* (Singer, 1925) while with streptococci (Hopkins and Parker, 1918) and *B. typhosus* (Parker and Franke, 1918) immunisation has no effect.

#### OUR EXPERIMENTS.

##### *Technique.*

Live cultures, 24 hours old, grown on agar at 37° C., were suspended in normal saline, and 1 c.c. of the suspension was injected intravenously into rabbits, because these animals are so susceptible to the action of this organism. Formol-killed vaccines of about the same density as the live suspensions of *S. aureus* were used in a similar manner, and also equal parts of Higgin's waterproof indian ink and normal saline filtered through several thicknesses of filter paper so as to obtain a uniform suspension free from large particles. In two instances a 20 per cent. solution of colloidal silver was used. Rabbits were inoculated in the marginal vein of the ear and killed at the required time interval by a blow on the neck. The organs were removed as quickly as possible after death and portions were put into Kaiserling solution and finally embedded in paraffin. Sections were stained with haemalum and eosin and also by Gram Weigert followed by haemalum and eosin.

##### *The distribution of staphylococci (S. aureus) and indian ink in the tissues, 5 minutes after intravenous inoculation.*

Live agar cultures, 24 hours old, of haemolytic and non-haemolytic *S. aureus*, and their vaccines, were used in all our experiments, but the same results were obtained in each case. When rabbits were inoculated intravenously

the most important microscopic changes were met with in the *lungs*. The alveolar walls were packed with polymorphs actively phagocytosing the cocci which were present in large numbers. The vessels were dilated and full of blood. The liver sinuses contained occasional polymorphs, and a few free cocci were seen. In the spleen, polymorphs were collected in clumps, but the cocci were few in number in the sinuses and mostly free. Cocci were scarce in the kidneys, but were found chiefly in polymorphs in the glomerular tufts. There was a very considerable difference in the experiments made with indian ink. The alveolar walls in the lungs were packed with empty polymorphs, but the ink particles were present in large amount, and phagocytosis of the ink by the large mononuclear cells had commenced. In the liver, however, considerable phagocytosis of the ink particles in the sinuses by the endothelial cells had taken place. The splenic sinuses were full of clumps of empty polymorphs and ink particles which were being engulfed by the endothelial cells. There was also a large amount of free ink in the glomerular and intertubular vessels in the kidneys.

Similar experiments were made at intervals of 20 minutes and 2 hours. The lungs of the *S. aureus* rabbits showed large numbers of polymorphs in the alveolar walls, and a few cocci phagocytosed by polymorphs and endothelial cells. In the liver sinuses large numbers of free cocci were found, and there was also phagocytosis by polymorphs and endothelials. In the spleen free cocci were numerous in the sinuses, and some were phagocytosed by polymorphs. There were very few polymorphs in the kidneys and very few cocci. With indian ink there was very little left in the lungs; mostly phagocytosed by the large mononuclears in the alveolar walls. In the liver, the ink was mostly in the endothelials in the sinuses. There was a massive collection of ink particles in the splenic sinuses, free and engulfed by endothelials. Very few ink particles were found in the kidneys, either free or in the endothelial cells. They were found chiefly in the glomeruli.

The examination of the tissues of a rabbit, killed 7 hours after the intravenous injection of *S. aureus*, showed a few cocci in the lungs and numerous polymorphs. Large numbers of cocci were found in the liver and spleen, where there was accumulation of polymorphs. The kidneys were of great interest. Polymorphs had collected in the glomeruli and in the capillaries of the intertubular tissues, but staphylococci were only found in clumps at the ends of the collecting tubules close to the pelvis.

The only polymorphs in the tissues of a rabbit killed after 22 hours were collected in the glomeruli of the kidneys. There were large numbers of free cocci in the liver sinuses, liver cells and in endothelial giant cells in the liver. In the kidney the staphylococci were present in clumps at the ends of the collecting tubules near the pelvis, and in the subepithelial connective tissue of the pelvis. Dudgeon and Goadby (1930) showed that, in rabbits which had died from *S. aureus* infections, various kidney lesions may occur, but especially (1) a simple acute pyelitis without any obvious lesion in the kidney

tubules; (2) linear abscesses radiating out from the papillae, apparently having started low down in the medulla at the end of the collecting tubules; (3) the whole kidney may be riddled with abscesses localised in the cortex or of the radiating linear type.

*Effect of previous immunisation.*

Rabbits were "immunised" by weekly injections of an *S. aureus* vaccine followed at a similar interval by two doses of the live organism, and were killed either 6 or 20 minutes after the last dose of the live coccus. In 6 minutes an enormous number of polymorphs had collected in the viscera, especially in the lungs, but not many cocci were found, although phagocytosis by the polymorphs was well shown. After 20 minutes similar polymorph accumulation was present, but many more cocci were visible in the lungs, liver and spleen. It would appear possible from our experiments that the effect of previous immunisation is to increase the immediate collection of polymorphs in the capillaries of the lungs and other organs.

*On the injection of "supernatant" saline suspensions of S. aureus.*

A haemolytic strain of *S. aureus* was grown on agar for 24 hours. The growth was washed off with sterile normal saline, and the suspension shaken for 30 minutes and finally centrifugalised until the supernatant fluid was clear and apparently free of cocci. One c.c. of this clear fluid was injected intravenously into two rabbits, which were killed after 5 and 20 minutes respectively. There was very great cardiac dilatation, engorgement and dilatation of small vessels in the internal organs, and polymorphs had accumulated in the lungs, liver and spleen. In the animals killed 20 minutes after inoculation the venous engorgement had resulted in considerable oedema in the heart muscle and in the liver. Rabbits were injected also with agar cultures of *S. aureus* grown for 24 hours at 37° C. and then suspended in the supernatant saline previously referred to, and killed 5 and 20 minutes later. In these rabbits, although the usual accumulation of polymorphs was present in the lungs, the cocci were not held up there, but were all over the body especially in the liver. Polymorph phagocytosis was active in all the organs, and it is probable that the great vascular dilatation was responsible for the rapid distribution of the cocci. The inoculation of dead staphylococci suspended in this supernatant fluid produced a similar reaction to the saline suspensions. Rabbits inoculated with live *S. aureus*, together with a suspension of washed human red cells, showed the same polymorph accumulation and phagocytosis of the cocci as occurred in the control experiments without red cells, and phagocytosis of the red cells by endothelial cells.

*Action of free diphtheria toxin and toxin adsorbed to Indian ink.*

(a) Within 5 minutes of an intravenous injection of diphtheria toxin into rabbits an accumulation of polymorphs of an unusual type was found in the

lungs, liver and spleen. These cells, instead of containing eosinophil granules characteristic of the polymorphs of rabbits, showed a hyaline non-granular cytoplasm or faintly stained eosin granules.

(b) A suspension of indian ink in diphtheria toxin to a concentration of 1 in 5 was left at 0° C. for 24 hours, and part of the mixture was autoclaved at 128 lb. for 30 minutes. Each preparation was shaken at short intervals.

One c.c. of each suspension was injected intravenously into rabbits. Sections of the viscera showed the same aggregations of ink in the capillaries and phagocytosis by endothelial cells, as in the control experiments with indian ink suspended in saline. The toxin had not stimulated the polymorphs to phagocytose the ink particles, and the polymorphs in the spleen and lung near the clumps of ink contained the usual eosin granules.

#### THE BLOOD PLATELETS.

Our results show that, when a massive dose of foreign particles is injected into the blood stream, they are quickly agglutinated, together with the platelets, which are thus removed from the circulating blood, and the number of platelets lost would appear to depend on the number of foreign particles injected. The platelets are rapidly restored again to their normal numbers, however, after an injection of colloidal silver and indian ink. This may be due to the breaking up of the clumps and not to a fresh output of platelets from the bone marrow. When *S. aureus* is injected in the circulation the platelets seem to be completely removed, for they are not seen in clumps in the shed blood, and take much longer to return to their normal numbers than when ink or colloidal silver is used. Bull (1915) states that when anti-pneumococcal serum is injected intravenously into animals infected with pneumococci, agglutination of the organism occurs in the blood stream, and subsequently phagocytosis by polymorphs in the lungs, liver and spleen. He found experimentally *in vitro* that when the immune serum was added to live pneumococci suspended in defibrinated blood, agglutination occurred at a higher titre than with saline suspensions of the organism. Bull found also that the action of an agglutinating serum is much more rapid and acts at a considerably higher dilution when injected intravenously than when tested *in vitro*, and is equally type specific. Delrez and Govaerts (1918) found that when anti-pneumococcal serum is injected intravenously it causes agglutination of the pneumococci, but they do not clump with the platelets.

We attempted to ascertain whether, in a convalescent case of paratyphoid fever, the patient's platelets had acquired the power of sticking to and causing the agglutination of the specific organism. Blood was taken rapidly from a vein by an ordinary needle (not waxed) direct into sodium citrate solution in a small waxed centrifuge tube, so that the concentration of citrate was 1 per cent. This citrated blood was centrifugalised and the supernatant plasma containing the platelets which was used for our experiments is referred to below as "platelets." Serum was taken at the same time, and was found to

have an agglutinating titre of 1/1000 with our stock *B. paratyphosus* B antigen. A 24 hours' agar culture of *B. paratyphosus* B and of a haemolytic *S. aureus* grown at 37° C. were suspended in normal saline. Agglutination tests were then made in waxed tubes at 37° C. as follows:

- (1) "Platelets" alone.
- (2) "Platelets" and the patient's serum.
- (3) "Platelets" and *B. paratyphosus* B.
- (4) "Platelets" and *S. aureus*.
- (5) "Platelets" and *B. paratyphosus* B and patient's serum.
- (6) "Platelets" and *S. aureus* and patient's serum.
- (7) *B. paratyphosus* B and patient's serum.
- (8) *S. aureus* and patient's serum.

The total volume in each tube was 1 c.c. 0.5 c.c. "platelets" was put into the tubes with a waxed pipette. The serum was diluted 1 in 10. At the end of 2 hours at 37° C. there was good agglutination in the control tube (7) containing *B. paratyphosus* B antigen and serum. Drops were then taken in waxed pipettes from the other tubes and examined as follows:

- (1) Hanging drop.
- (2) Dried and stained with Leishman.
- (3) Smear made and stained with Leishman.
- (4) Mixed directly with cresyl blue solution and allowed to dry; counter-stained with Leishman.

Each preparation showed a similar result:

- (a) The platelets were all quite separate and had not clumped in any of the tubes.
- (b) Those bacteria, which were not agglutinated, were not adherent to the platelets.
- (c) Small and large clumps of bacteria were quite separate from the platelets.

This experiment shows that the platelets had not acquired the property of adhering to the specific organisms; they were not clumped and had not agglutinated the bacilli, and that the conglomeration of organisms and platelets which occurs in whole blood in the circulation had not occurred in test-tube experiments such as we have described.

Wright (1927) by *in vitro* experiments showed that a suspension of indian ink, which is stable in saline and blood serum, is precipitated by whole blood in very small particles, some of which are aggregated with platelets.

It is as yet not proven that the platelets are important in the elimination of foreign particles from the circulation.

Delrez and Govaerts (1918) observed a big drop in the circulating platelets following the injection of *S. aureus* or *B. paratyphosus* B as the bacteria became attached to clumps of platelets.

Govaerts (1921) stated that an injection of anti-platelet serum before an animal is inoculated with *B. typhosus* has no effect on the rate of disappearance of the bacillus from the blood stream, and Bull and McKee (1922) confirm these results with *S. aureus* and pneumococci. Taniguchi, Joogetsu and Kasahara (1930) found a rapid disappearance of platelets from the peripheral blood after the intravenous injection of various organisms, but the number of platelets returned to the normal in 1 hour. They observed agglutination of bacteria and platelets in films made from the blood of infected animals.

Using the technique recently described by Goadby (1930) platelets were counted in rabbits before, and at various intervals after, the intravenous injection of live and dead *S. aureus*, indian ink and colloidal silver. The blood examinations were made 1½, 5 and 10 minutes, and 2 hours, after the inoculations. In Table I is shown the effect of the intravenous inoculation of live and dead *S. aureus*. The very rapid fall of blood platelets immediately after the injection of the living organisms or a massive dose of the dead coccus is a very striking phenomenon. Two hours after the injection there is a considerable rise in the number of platelets, although not to the same height as recorded previous to inoculation.

Table I. *The number of platelets per c.mm. of blood, before and after the intravenous inoculation of S. aureus.*

	1 Live organism	2 Vaccine 3000 million	3 Vaccine 30,000 million
Before inoculation	503,400	489,000	1,009,200
Time after inoculation			
1½ minutes	121,000	425,000	187,000
5 minutes	89,400	355,000	184,200
10 minutes	124,000	525,000	356,400
2 hours	364,800	438,000	445,000

After the intravenous inoculation of 20 per cent. colloidal silver, the immediate fall in platelets was even greater than from live *S. aureus*, but amorphous masses of silver and platelets could be seen clumped together, so that the platelets were not countable because the number of free platelets was insignificant. With indian ink, clumps of ink and platelets occurred, but it was possible to count the platelets in the clumps with a fair degree of accuracy. It was noted that the fall in the platelets was very slight and in no sense comparable to the fall following the inoculation of live *S. aureus*.

The results of some experiments done by us suggest that the agglutination of ink and platelets actually occurs in the circulating blood, and not merely in the platelet counting bottle after the blood has been taken for examination, and similarly with colloidal silver. The platelet count in a normal rabbit was done by Goadby's technique, by which 40 c.mm. of blood are taken into a waxed bottle. A small amount of indian ink was then added to the diluted blood in the bottle, so that the concentration of the ink was approximately the same as in the blood of a rabbit injected intravenously with 1 c.c. of

indian ink. The mixture was shaken from time to time for a period of 2 hours, and then the platelets were counted to see whether there was any diminution in their numbers or if they had clumped together with the ink, as occurs after an intravenous injection. It was found that the platelet count was unaltered and that no clumping of the ink and platelets had occurred.

#### CONCLUSIONS.

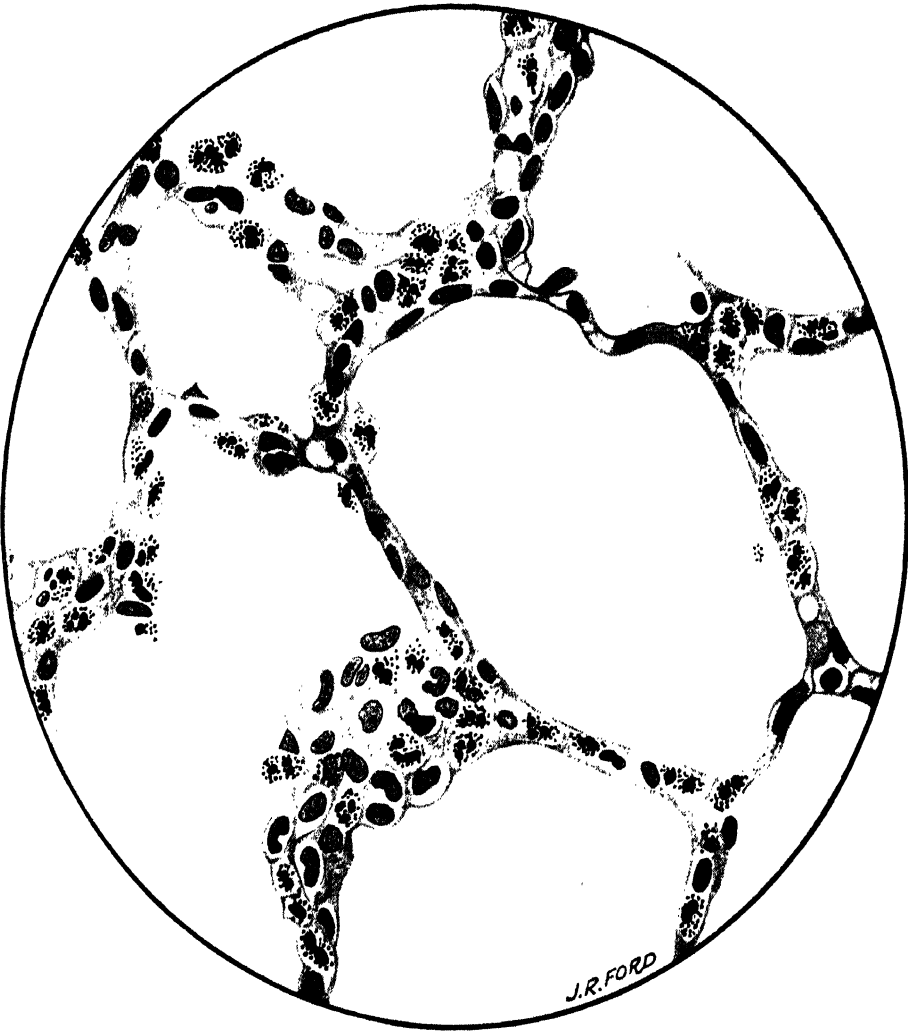
When any inert suspension such as indian ink or colloidal silver is injected into the circulation, the first effect is the aggregation of the particles with the platelets: these clumps adhere to the walls of the lung capillaries first, some pass on to the liver and spleen sinuses and, in these situations, the foreign particles are taken up by the endothelial cells. At the same time, nearly all the circulating polymorphs collect in the capillaries of the internal organs, *especially in the lungs*, but there is little phagocytosis by these cells. As the particles are removed from the circulation, the platelets are restored to the blood, probably by the breaking up of the clumps, but possibly also by a fresh supply from the bone marrow. There are always in any situation a certain number of particles not ingested, and also a number of empty phagocytes, however much excess of foreign body is present.

When a suspension of *S. aureus* is injected intravenously the same process develops. The cocci are conglomerated with the platelets, but these clumps are filtered off at first almost entirely in the lung capillaries where the available polymorphs collect, and almost instantaneously active phagocytosis of the cocci occurs. This is in great contrast to indian ink which is quickly distributed all over the body and taken up by the endothelial cells. When ink or colloidal silver is injected intravenously, the blood is observed to be very dark in colour within 30 seconds.

The polymorphs containing the ingested cocci may pass further on into the circulation, and be arrested in the spleen or other organs; some migrate through the alveolar walls and can be seen in the lumen of the alveoli or bronchi; others are phagocytosed by the endothelial cells. As time goes on, more and more cocci are found free in the capillaries in spite of the presence of large numbers of polymorphs which are not phagocytic.

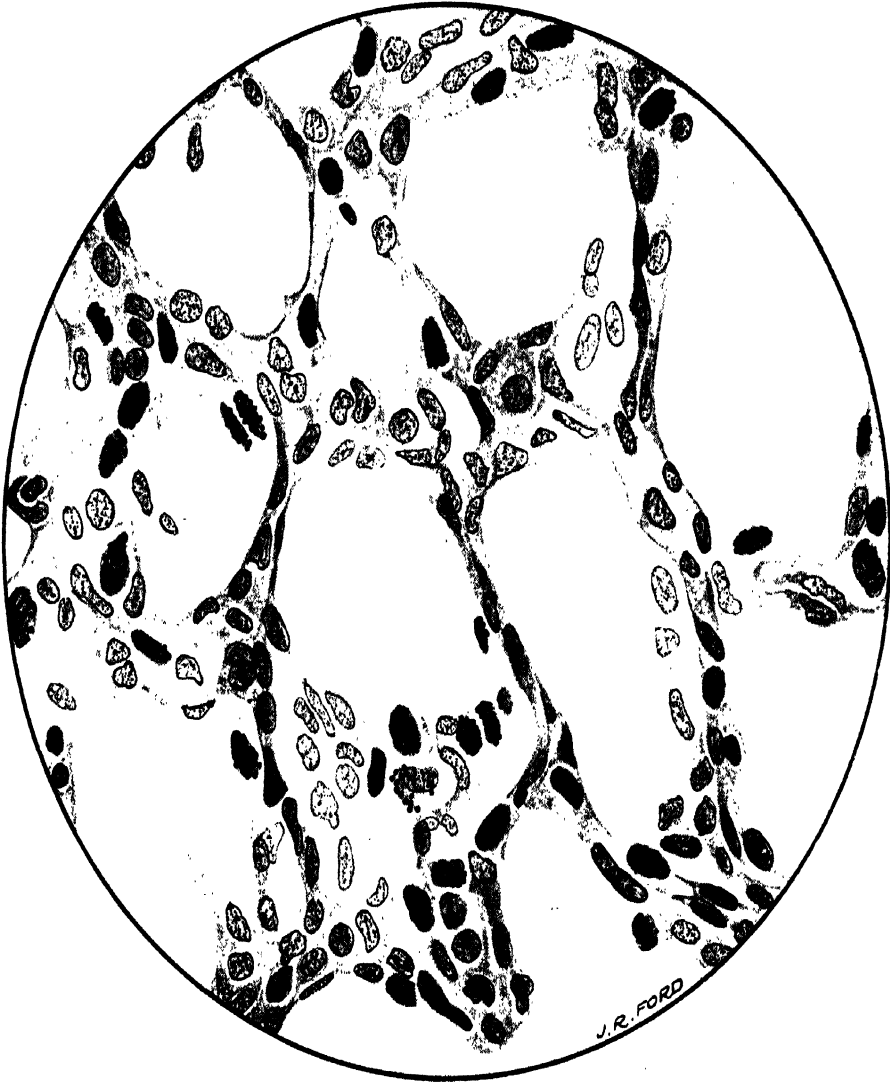
#### SUMMARY.

1. The tissue reactions in rabbits from intravenous injections of live and dead *Staphylococcus aureus* and massive doses of indian ink and colloidal silver have been studied.
2. Any particles injected into the circulation cause the accumulation of polymorphs in the lung capillaries.
3. Inert colloidal particles such as indian ink are clumped in the capillaries of the lungs, liver, spleen and kidneys, and are phagocytosed by the endothelial cells.

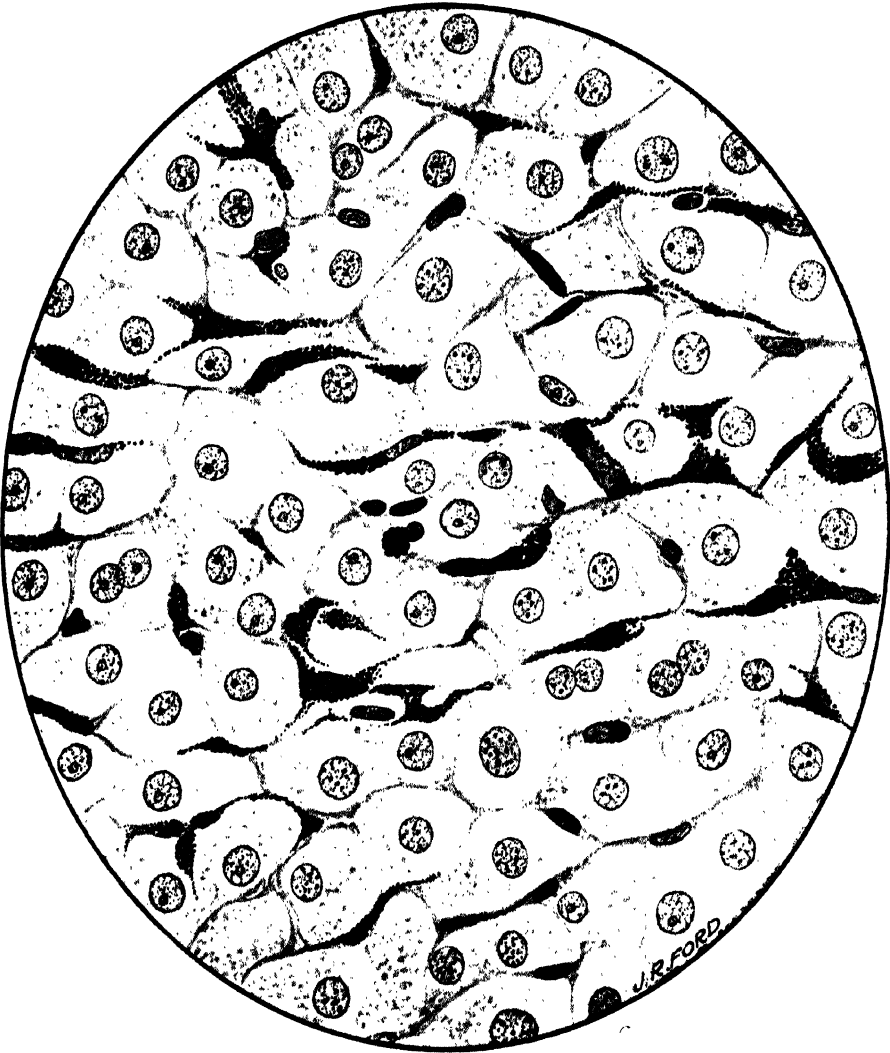


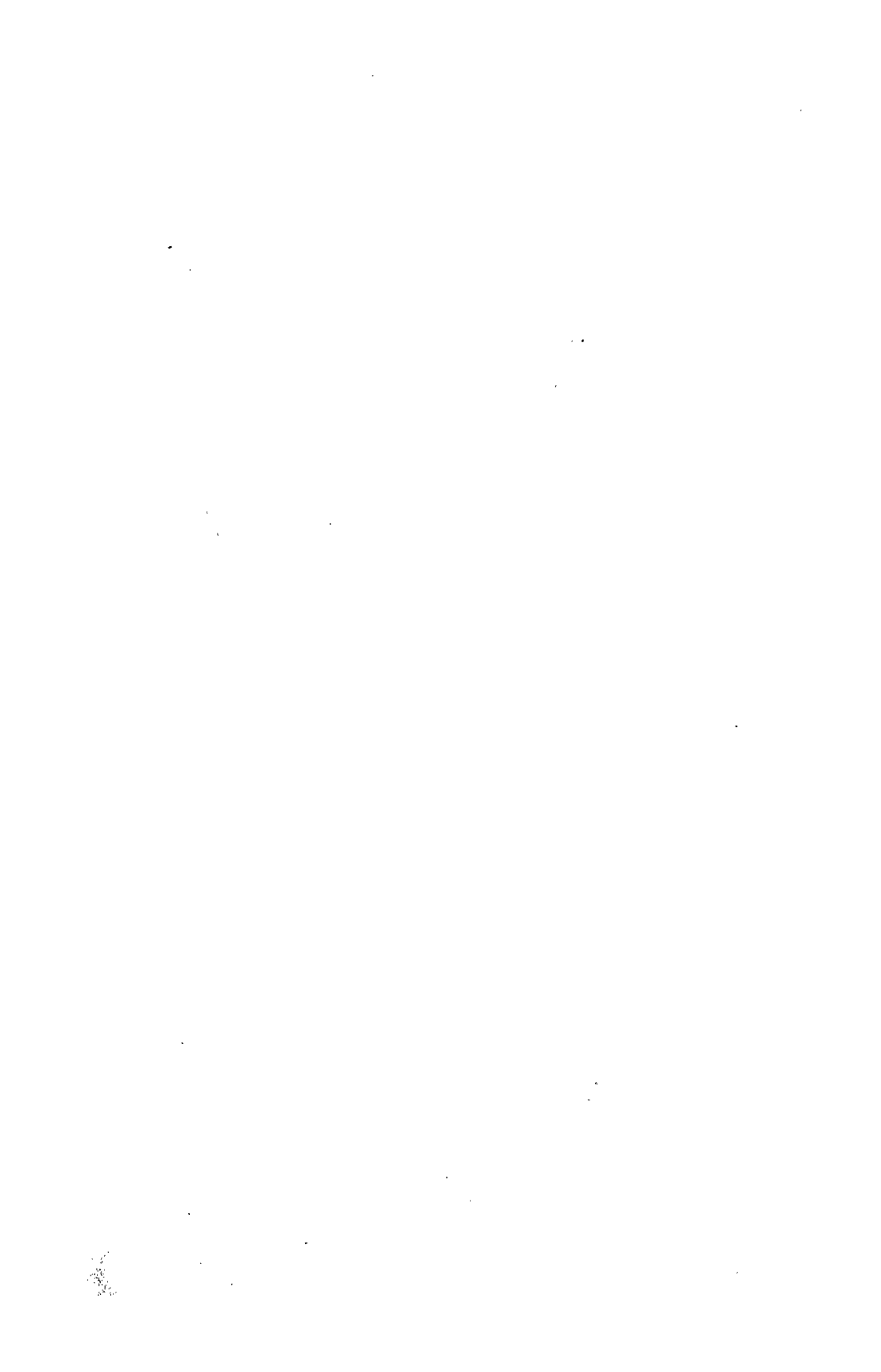












4. Staphylococci (*S. aureus*), live or dead, are nearly all held up in the lungs, where they are actively phagocytosed by the polymorphs within 5 minutes of an intravenous injection.

5. Subsequently the cocci are distributed to the other organs, where phagocytosis continues mainly by polymorphs, but in the liver also by the Kupfer cells.

6. Special attention is drawn to the localisation of the cocci in certain areas in the kidneys.

7. Platelet counting on animals injected with various substances showed that there is an agglomeration of the particles with the platelets, which are consequently removed from the circulation.

8. In the case of the inert particles the platelets are then restored to the circulation. With organisms (*S. aureus*) some of the platelets appear to be completely removed from the blood together with the bacteria.

## EXPLANATION OF PLATES I-III.

### PLATE I.

Lung of rabbit. Polymorphs in alveolar walls 5 minutes after an intravenous inoculation of *S. aureus*. Section stained with haemalum and eosin. ( $\times 650$ .)

### PLATE II.

Lung of rabbit. Polymorphs in alveolar walls showing very active phagocytosis of *S. aureus* 5 minutes after an intravenous inoculation of this organism. Section stained with haemalum and Gram's stain. ( $\times 650$ .)

### PLATE III.

Liver of rabbit. Large mononuclear cells in liver sinuses showing very active phagocytosis of indian ink particles 5 minutes after an intravenous inoculation of this substance. Sections stained with haemalum and eosin. ( $\times 650$ .)

## REFERENCES.

- ANDREWES, F. W. (1910). *Lancet*, i, 1737; ii, 8, 83, 153.  
 BAIL, O. (1905). *Arch. f. Hyg.* 52, 272.  
 BARTLETT, C. J. and OZAKI, Y. (1917). *J. Med. Res.* 35, 465.  
 — (1918). *Ibid.* 37, 139.  
 BOERNER-PATZELT, D. (1923). *Zeitschr. f. d. ges. exper. Med.* 34, 336.  
 BULL, C. (1914). *J. Exper. Med.* 20, 237.  
 — (1915). *Ibid.* 22, 457, 466, 475, 484.  
 — (1916). *Ibid.* 24, 7, 25.  
 BULL, C. and MCKEE, H. (1922). *Amer. J. Hyg.* 2, 208.  
 CAPPELL, D. F. (1930). *J. Path. and Bact.* 33, 175-96.  
 DELREZ, L. and GOVAERTS, P. (1918). *C. R. Soc. Biol.* 81, 53.  
 DUDGEON, L. S. and GOADBY, H. K. (1930). *J. Hygiene*, 30, 180.  
 GOADBY, H. K. (1930). *J. Path. and Bact.* 33, 1171.

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- GOVAERTS, P. (1919). *C. R. Soc. Biol.* **82**, 927.  
—— (1920). *Ibid.* **83**, 196.  
—— (1921). *Ibid.* **85**, 667.  
HOPKINS, J. G. and PARKER, J. T. (1918). *J. Exper. Med.* **27**, 1.  
LANG, F. J. (1926). *Arch. Path. and Lab. Med.* **1**, 41.  
LEVADITI, C. (1901). *Ann. Inst. Pasteur*, **15**, 894.  
NAGAO, K. (1920). *J. Inf. Dis.* **27**, 327.  
NISSEN, R. (1922). *Zeitschr. f. d. ges. exper. Med.* **28**, 193.  
PARKER, J. T. and FRANK, E. (1918). *J. Med. Res.* **39**, 301.  
SINGER, E. (1925). *Zeitschr. f. Immunitätsforsch.* **43**, 285.  
TANIGUCHI, T., JOOGETSU, M. and KASAHARA, T. (1930). *Japan J. Exper. Med.* **8**, 55.  
WERIGO (1894). *Ann. Inst. Pasteur*, **8**, 1.  
WESTHUES, M. (1922). *Beitr. z. allg. Path. u. Path.-Anat.* **70**, 223.  
WISLOCKI, S. B. (1924). *Amer. J. Anat.* **32**, 423.  
WRIGHT, H. D. (1927). *J. Path. and Bact.* **30**, 185.

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## CONTRIBUTIONS TO THE EXPERIMENTAL STUDY OF EPIDEMIOLOGY.

### THE EFFECT OF VACCINATION ON HERD MORTALITY.

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IN several previous reports we have described the behaviour of communities of mice, submitted over long periods of time to the risks attendant on the epidemic prevalence of a bacterial infection. These communities have been recruited in ways varying both as regards the rate of immigration and the nature of the immigrants.

The present report deals with an experiment in which we have studied the effect of prophylactic immunisation, with a suitable bacterial vaccine, in modifying the course of events in our experimental herds.

As in many of our previous experiments, the infection selected for study was mouse typhoid, due to infection with *Bact. aertrycke*. There is abundant evidence that the active immunisation of mice with killed suspensions of this organism results in an increased resistance to subsequent experimental infection with living cultures (Loeffler, 1906; Wolf, 1908; Yoshida, 1909; Brückner, 1911; Webster, 1922; Ornstein, 1922; Neufeld, 1924; Lange and Yoshioka, 1924; Topley, Wilson and Lewis, 1925). The immunity so induced has, however, usually been of a low order. A proportion of the immunised mice have resisted a subsequent experimental infection, and those which have succumbed during the experimental period have, on the average, lived longer than the unvaccinated controls, but fatal infections have not been prevented, and, in most experiments, a considerable proportion of the vaccinated mice have died from the disease. Some evidence has also been obtained that such active immunisation may exert an inhibitory effect on the epidemic spread of mouse typhoid (Lynch, 1922; Topley and Wilson, 1923; Topley, 1926). The few adequately controlled experiments so far recorded deal only with epidemics running their course in closed communities; and the results suggest that, even under these conditions, immunisation must be carried out *before* exposure to infection, and must be applied to the *whole* of the population at risk, if any significant degree of herd protection is to be attained. Our main object, in the experiment here reported, has been to provide data which will allow us to compare the behaviour of normal with that of artificially immunised mice during the course of a long-continued epidemic, and, in particular, to compare as far as possible the effect of artificial immunisation before admission to the infected herd with that of the natural immunisation, which occurs during the



earlier period of exposure to risk. The opportunity has been taken of assessing the relative value of different bacterial vaccines, and an attempt has been made to compare the immunising effect of various antigenic components, and to test the prophylactic value of purely non-specific antigens.

The work of Felix and his colleagues (Felix, 1924; Robertson and Felix, 1930), of Arkwright (1927), of Ibrahim and Schütze (1928) and of Schütze (1930) has already provided a considerable body of evidence in favour of the view that the somatic antigens of the normal smooth form of bacilli of the typhoid-paratyphoid group (the so-called O antigens) are far more significant immunologically than the flagellar (H) antigens, or than the somatic antigens of rough variants (the so-called  $\phi$  or R antigens). As will be seen, our own results are entirely confirmatory of this view. A brief account of this aspect of our enquiry, dealing only with the earlier phases of our experience, has already been recorded elsewhere (Topley, 1929).

One other point in relation to antigenic structure has been investigated. Schütze (1928) has noted that one serological type of *Past. pseudotuberculosis* possesses a somatic antigen which is related to that of *Bact. aertrycke*. Such relationships are of obvious importance in epidemiological enquiries. Antigenic specificity is fundamentally chemical in nature, and only specific in the bacteriological sense as a result of the actual distribution of antigenic components among different bacterial species. It would accord quite well with our knowledge of immunity in general to find that infection, or immunisation, with one bacterial species, increased resistance of the host to any other pathogenic bacterium, which shared with it an immunologically significant antigenic factor.

As a preliminary to the description of the happenings in our herd of mice, we note the nature of the vaccines employed, and certain additional data with regard to their immunological properties.

*Vaccine A.* This consisted of a saline suspension of *Staphylococcus albus*, killed by the addition of 0.25 per cent. formalin followed by heating at 55° C. for 1 hour. A single batch of vaccine was prepared shortly before the commencement of the experiment, and was employed throughout the period l. v. 28 to 27. xi. 28. This vaccine was selected as an example of an entirely non-specific immunising agent. The mice inoculated with this vaccine are referred to as the *A* mice.

*Vaccine B.* A suspension of a rough strain of *Bact. typhosum* in saline containing 0.25 per cent. formalin, steamed at 100° C. for 30 minutes. Selected as a material containing the cosmopolitan rough somatic antigen, but neither the H nor O antigens of the normal smooth *Bact. aertrycke*. One suspension was used from l. v. 28 to 27. xi. 28. The mice inoculated with this vaccine are referred to as the *B* mice.

*Vaccine C.* A suspension of a smooth strain of *Bact. aertrycke* in 0.25 per cent. formol saline, heated at 55° C. for 1 hour. This vaccine contained organisms in the type and group phase, and thus contained the O antigen and the two types of H antigen corresponding to the two alternative phases. One suspension was used from l. v. 28 to 9. vii. 29. The mice inoculated with this vaccine are referred to as the *C* mice.

*Vaccine D.* A suspension of a completely rough strain of *Bact. aertrycke*, prepared in 0.25 per cent. formol saline, and heated at 55° C. for 1 hour. It contained the R, or  $\phi$ ,

rough somatic antigen and the H antigen of the type and group phases, but did not contain the smooth somatic antigen in detectable amount. One suspension was used from 1. v. 28 to 27. xi. 28. The mice inoculated with this vaccine are referred to as the *D* mice.

*Vaccine E.* A broth culture of *Bact. aertrycke*, in the normal smooth phase, incubated for 24 hours at 37° C. and then killed by the addition of 0.25 per cent. formalin, followed by heating to 55° C. for 1 hour. It contained the O antigen, and the H antigen in the type and group phase. One suspension was used from 1. v. 28 to 27. xi. 28. The mice inoculated with this vaccine are referred to as the *E* mice.

*Vaccine F.* A saline suspension of *Bact. paratyphosum* B smooth in the group phase, killed by the addition of 0.25 per cent. formalin followed by heating at 55° C. for 1 hour. It contained the O antigen, common to *Bact. paratyphosum* B and to *Bact. aertrycke*, and the H antigen shared by these species in the group phase. One suspension was employed between 1. v. 28 and 27. xi. 28. The mice inoculated with this vaccine are referred to as the *F* mice.

*Vaccine G.* Similar to *F*, except that the strain selected was in the type phase. The only antigenic constituent of this vaccine represented in *Bact. aertrycke* was, therefore, the O somatic antigen. One suspension was used between 1. v. 28 and 27. xi. 28. The mice inoculated with this vaccine are referred to as the *G* mice.

*Vaccine J.* A saline suspension of *Past. pseudotuberculosis*, of the type showing a serological relationship to *Bact. aertrycke*<sup>1</sup>, killed, as above, with formalin and heat. The antigenic structure of this strain will be briefly considered below. A single suspension was used between 4. xii. 28 and 5. ii. 29. The mice inoculated with this vaccine are referred to as the *J* mice.

*Vaccine K.* A saline suspension of *Bact. aertrycke*, prepared in January 1929, in exactly the same way as vaccine *C*, and containing the same antigenic factors. One suspension was used between 19. ii. 29 and 9. vii. 29. The mice inoculated with this vaccine are referred to as the *K* mice.

In every case the suspension to be inoculated was diluted to contain  $1000 \times 10^6$  bacilli per c.c., and 0.5 c.c. of this was injected intraperitoneally. One week later the injection was repeated, using the same dose, and the mice were added to the experimental cage on the seventh day after the second inoculation.

An experimental epidemic of mouse typhoid was started on 4. i. 28, by infecting fifty mice with *Bact. aertrycke*, adding to them fifty normal mice and, thereafter, adding three normal mice a day until the conclusion of the experiment (17. ix. 29). The inoculated mice, corresponding to the various vaccines employed, were added in batches every seventh day, and with them were added a numerically equal batch of normal mice, referred to hereafter as the *N* or normal mice.

Thus, from 1. v. 28 to 27. xi. 28, ten mice of each of the vaccinated groups *A*, *B*, *C*, *D*, *E*, *F*, *G*, and ten normal mice were added to the cage every seventh day.

From 4. xii. 28 to 5. ii. 29, twenty *C* mice, twenty *J* mice, and twenty normal mice were added to the cage every seventh day.

From 19. ii. 29 to 9. vii. 29, twenty *C* mice, twenty *K* mice, and twenty *N* mice were added to the cage every seventh day.

The addition of three normal mice a day was continued until 17. ix. 29 in

<sup>1</sup> For a culture of this strain we are indebted to Dr Schütze.

order to keep the conditions as comparable as possible until the last of the vaccinated mice added had survived in the cage for 60 days.

Thus, during the period 1. v. 28 to 27. xi. 28, we were able to compare the immunising values of: (1) a non-specific stimulus—Group *A*; (2) the rough somatic antigen alone—Group *B*; (3) the rough somatic antigen combined with the H antigen in both the type and group phase—Group *D*; (4) the smooth O antigen alone—Group *G*; (5) the smooth O antigen combined with the H antigen of the group phase—Group *F*; (6) the complete antigenic combination (Smooth O + H type + H group)—Group *C*; and (7) the same combination together with any other antigenic constituent which might be present in a broth culture, but absent from a saline suspension—Group *E*.

During the period 4. xii. 28 to 5. ii. 29 we were able to compare the immunising value of a *Past. pseudotuberculosis* vaccine (Group *J*) with that of a *Bact. aertrycke* vaccine (Group *C*).

During the period 19. ii. 29 to 5. vii. 29 we were able to compare the immunising value of a freshly prepared *aertrycke* vaccine (Group *K*) with that of a similar vaccine which was some 15 months old at the commencement of this period (Group *C*).

The data for the whole period are, of course, available for our main object—the comparison of the fate of artificially immunised mice with that of normal mice (*a*) on entry to the cage, and (*b*) after various periods of exposure to risk.

Certain ancillary experiments have been carried out, and certain additional observations have been made, to determine more exactly the nature of the reagents which we have employed. These may be summarised as follows.

#### THE INHERENT TOXICITY OF THE VACCINES EMPLOYED.

Certain of the vaccines possessed an appreciable toxicity for mice in the doses in which they were administered, so that a proportion of the mice died during the 14 days elapsing between the inoculation of the first dose of vaccine and addition to the cage. In order to have sufficient mice available for addition we inoculated twelve to fifteen of each batch, instead of the ten actually required.

The vaccination death-rate for the various groups is shown in Table I.

Table I.

Group	Total no. vaccinated	Deaths during 14 days following first vaccination	Percentage mortality
<i>A</i>	390	10	2.56
<i>B</i>	403	13	3.23
<i>C</i>	1224	96	7.84
<i>D</i>	396	17	4.29
<i>E</i>	423	44	10.40
<i>F</i>	398	30	7.54
<i>G</i>	394	13	3.30
<i>J</i>	240	13	5.42
<i>K</i>	509	45	8.84

THE ANTIGENIC CHARACTER OF THE VARIOUS VACCINES EMPLOYED,  
AS JUDGED BY THE ANTIBODY RESPONSE IN MICE.

The vaccination of a number of mice in excess of that required for addition to the cage left us, each week, with a small number of surplus vaccinated mice. These were anaesthetised and bled from the jugular vein. The serum so obtained was tested against formalinised broth suspensions of *Bact. aertrycke* in the group and type phase to determine the titre of H agglutinins, and against an alcoholised suspension of *Bact. aertrycke* to determine the titre of O agglutinins. In the case of Groups *A* to *G*, the majority of the agglutination tests were carried out at a commencing titre of 1:20. Table II shows the number of sera tested in each group, and the number of the sera which agglutinated these suspensions to a titre of 1:20 or over. It may be noted that a high proportion of the sera showed a titre of 1:640 (the highest dilution tested) against the H suspensions, while no serum showed a titre of 1:160 or over against the O suspension.

Table II.

Group	No. of sera tested	No. of sera agglutinating different suspensions of <i>Bact. aertrycke</i> at 1:20 or over			
		H		O and H	O alone
		Type	Group		
<i>A</i>	70	0	0	0	0
<i>B</i>	72	0	0	0	0
<i>C</i>	60	58	59	4	0
<i>D</i>	61	59	36	0	0
<i>E</i>	66	65	64	5	0
<i>F</i>	52	0	52	10	0
<i>G</i>	62	0	0	0	5

It will be seen that the vaccines behaved in the way which was expected. The staphylococcus vaccine (*A*) showed itself entirely devoid of the antigenic factors with which we are concerned. The rough typhoid vaccine (*B*) was equally devoid of these factors. (The presence of agglutinins acting on the rough somatic antigen was not determined.) The rough *aertrycke* vaccine *D* stimulated the formation of H antibodies, but not of O antibodies. The paratyphoid B vaccine in the type phase (*G*) stimulated the formation of O antibodies alone, so far as *Bact. aertrycke* was concerned. A similar vaccine in the group phase gave rise to O antibodies, and to H antibodies of the group variety. The *aertrycke* vaccines *C* and *E* caused the formation of both H and O agglutinins.

It will be obvious that the production of O agglutinins is, in all cases where both are formed, far less copious than that of H agglutinins. It seems to us probable, in view of the results to be described later, that it would be unjustifiable to assume that the production of O antibodies is, in fact, confined to those mice in which agglutinins acting on alcoholised suspensions are demonstrable in the serum. It seems more likely that the presence of such

agglutinins to a demonstrable titre in a proportion of the mice concerned should be taken as evidence that the corresponding stimulus has been provided, while it may well be that mice which show no such demonstrable agglutinins may have made a specific response which has altered their resistance to infection.

During the latter part of the experiment, the testing of sera against the formalinised suspensions was discontinued, in order that we might employ the small amount of serum available from each mouse in testing for O agglutinins at a lower titre (1:5). The results of these tests are set out in Table III.

Table III.

Group	No. of sera tested	No. of sera agglutinating an O suspension of <i>Bact. aertrycke</i> at 1:5 or over
<i>A</i>	8	0
<i>B</i>	8	0
<i>C</i>	118	8
<i>D</i>	8	0
<i>E</i>	3	0
<i>F</i>	6	0
<i>G</i>	8	1
<i>J</i>	27	0
<i>K</i>	44	2

The results are very similar to those obtained with a serum dilution of 1:20. The *Past. pseudotuberculosis* vaccine (*J*) shows no evidence of being able to stimulate the formation of O agglutinins in the mouse. The immunological properties of this vaccine are further considered below. We may add that we have tried to stimulate a more regular and copious formation of O agglutinins in mice by repeated injections of vaccines *C* or *E*, but without success.

THE IMMUNISING VALUE OF CERTAIN OF THE VACCINES EMPLOYED, AS TESTED BY THE INTRAPERITONEAL INJECTION OF LIVING *BACT. AERTRYCKE* IN VACCINATED MICE.

It seemed desirable to test the effectiveness for active immunisation against a subsequent single measured dose of virulent *Bact. aertrycke*, of certain of these vaccines which, as will be seen later, produced a significant change in the resistance to natural infection in our experimental cages. At the same time opportunity was taken of confirming the ineffectiveness of the *Past. pseudotuberculosis* vaccine. In each experiment the immunised mice received two doses of vaccine, each containing  $500 \times 10^6$  bacilli, with a week's interval between them. They were tested, 1 week after the second dose of vaccine, by the intraperitoneal inoculation of 1000 *Bact. aertrycke*, of a virulent strain. An equal number of normal unvaccinated mice were injected with the same dose and by the same route to serve as controls. All mice were kept under observation for 28 days. The results are summarised in Table IV.

Whether we take as our test of increased resistance the number of mice which survive for 28 days after the test inoculation, or the mean survival time

limited to 28 days, it is clear that a suspension of a smooth strain of *Bact. aertrycke*, killed by the addition of formalin followed by heating at 55° C. for 1 hour, is a relatively effective immunising agent, as judged by these particular experiments. The *Past. pseudotuberculosis* vaccine (*J*) is without appreciable effect.

Table IV.

Exp.	Vaccine	No. of mice	Average survival time—limited to 28 days	No. died within 28 days	No. survived 28 days	No. of survivors with positive spleen cultures
A	<i>C</i> (see above)	50	21.3	27	23	7
	<i>K</i> "	50	20.7	30	20	12
	<i>J</i> "	50	7.9	50	0	—
	Unvaccinated controls	50	7.1	49	1	1
B	Similar to <i>K</i>	50	22.4	29	21	12
	Unvaccinated controls	50	5.1	50	0	0
C	Similar to <i>K</i>	44	20.8	25	19	15
	Unvaccinated controls	50	5.2	50	0	—

With regard to the ineffectiveness of the *Past. pseudotuberculosis* vaccine as a prophylactic against infection with *Bact. aertrycke*, a few additional points may be noted. We have been able to confirm Schütze's observation, that there is an apparent antigenic relationship between these species, connected in some way with the O somatic antigen. Thus the serum of a rabbit immunised against *Past. pseudotuberculosis* agglutinated a suspension of that organism to 1:80, and an alcoholised suspension of *Bact. aertrycke* to the same titre. It also agglutinated an alcoholised suspension of *Bact. paratyphosum* B, which shares the same O antigen, but had no action on similar suspensions of *Bact. newport*, *Bact. suispestifer* or *Bact. enteritidis*, in which the O antigens are different. An agglutinating serum prepared against an O suspension of *Bact. aertrycke* agglutinated an alcoholised suspension of that organism to 1:5120, and a suspension of *Bact. pseudotuberculosis* to 1:40.

We have not succeeded in demonstrating any absorption of anti-aertrycke agglutinins by *Past. pseudotuberculosis*, or of anti-pseudotuberculosis agglutinins by *Bact. aertrycke*; but we have not pursued this problem beyond a single test, the results of which were entirely negative. The exact nature of the antigenic relationship between these two species has still to be finally determined, and it would, we think, be premature to assume that the characteristic O antigen of *Bact. aertrycke* is present in this type of *Past. pseudotuberculosis*. It may, however, be noted that the anti-pseudotuberculosis rabbit serum showed some power of protecting mice against experimental infection with *Bact. aertrycke*. This protection was of a low order. Only one of thirty mice survived the test inoculation, but those which died lived significantly longer than untreated controls, or than mice which had received normal rabbit serum. Moreover, the protection afforded was little if at all inferior to that given by the same dose of an anti-aertrycke serum containing

a high titre of O agglutinins. We may note that, in our hands, passive protection of mice against infection with bacilli of the enteric group, using rabbit antisera, has proved singularly ineffectual.

THE CONDITION OF THE IMMUNISED MICE WHICH SURVIVED SUBSEQUENT  
EXPERIMENTAL INFECTION.

A point of some importance is the actual condition of the immunised mice which survived until the twenty-eighth day. The results, in this respect, were confirmatory of all our previous experience. Taking all three experiments there were eighty-three such survivors. All were examined *post mortem*, and a portion of the spleen was transferred to broth. In forty-six cases a culture of *Bact. aertrycke* was obtained; in thirty-seven the broth remained sterile. With few exceptions these surviving mice showed no observable abnormality at necropsy, so that we must regard the survivors with positive spleen cultures as suffering from a persistent latent infection. All these mice were housed, from the day of the test inoculation onwards, in separate small cages, so that there was no possibility of re-infection from other infected mice. This procedure has always been followed by us in experiments of this kind. What would have happened if these mice had been maintained in isolation over much longer periods of time we are, of course, not in a position to say. We think it probable that some, at least, would have subsequently succumbed to an acute exacerbation of their infection, while others would in time have rid themselves of the bacilli which they were harbouring.

The thirty-seven negative spleen cultures suggest that some 19 per cent. of the immunised mice had their resistance increased to a point at which it was completely effective against a single dose of living *Bact. aertrycke*, in the sense that the tissues were freed entirely from living bacilli; but such figures as these may be quite misleading. It would require a detailed and laborious study of very large numbers of surviving mice to ascertain with any exactness the frequency of persistent but quiescent foci of infection throughout the tissues. The point is an important one, as will be seen when we come to describe the fate of the vaccinated mice in our infected herd. Are we to regard the reaction of an immunised mouse to the fluctuating dispersion of infective material within the cage as in the nature of a single decisive event, the first reception of a dose of infective matter being followed by a rapidly fatal illness, a persistent latent infection, or a complete return to the non-infected state, according to the size of the dose received and the resistance of the mouse? Or are we to regard this reaction as a series of events, not necessarily connected in any orderly sequence, of which the initial reception of infection forms merely the first chapter, and in which decisive happenings, of very varying nature, may occur at any period thereafter?

If the establishment of a persistent latent infection is a common reaction—and we have no doubt that it is—the subsequent events which determine ultimate death or recovery *may* have little connection with further risks of

specific infection in the cage. The mouse may have purchased immunity to such risks at the price of a heightened susceptibility to others, such as fatigue, dietetic influences, or other factors, which might upset the equilibrium on which this infection immunity depends, and precipitate a fatal illness. It should be noted, however, that this possible indifference to repeated doses of the specific infective material is by no means a necessary consequence of the view that latent infections are common. It is quite likely that a latently infected mouse might at one time be more resistant to specific infection than a normal animal, at another more susceptible.

If, on the other hand, the complete elimination of the infecting bacteria is a frequent sequence to a primary infection of the vaccinated mice, then the frequency and amount of subsequent doses of infective material will clearly be of preponderating importance, as will also any changes in the specific resistance of the host induced by the earlier doses which have been successfully eliminated.

In this connection, also, there falls to be considered a possible source of error in the interpretation of our experimental findings. We have, throughout our investigations, accepted the recovery of *Bact. aertrycke* from the tissues at the necropsy on any mouse as evidence that that mouse died from the enteric infection. No other criterion is, in fact, possible, since many of those mice which die of acute infection, yielding copious growths of *Bact. aertrycke* from the heart blood, show no characteristic lesions detectable by the naked eye. If the vaccinated mice in our experimental herds frequently contracted a persistent latent infection, which seldom led to a fatal result, they might conceivably be recorded as specific deaths, as a result of the persistence of living bacilli within their tissues, though they had in fact died from some quite different cause. The actual findings at necropsy showed quite clearly that this was not commonly the case, since the characteristic lesions of the disease were frequently present in the vaccinated mice; but, in order to determine whether the figures based on our usual criterion had, from this cause, been weighted to any degree against the vaccinated animals, we tabulated, for the later period of the experiment the frequency of the more significant *post mortem* findings of each of the groups exposed to risk. These are set out in Table V and are, we think, decisive.

Table V. *Showing the findings at necropsy in the various groups of mice.*

	H	Daily	J	K	C
Number of mice examined <i>post mortem</i>	468	660	143	317	518
Percentage of above from which <i>Bact. aertrycke</i> was not isolated	9.6	13.5	10.4	12.3	12.5
Number of specific deaths	423	571	128	276	453
Percentage of above in which <i>Bact. aertrycke</i> was isolated from spleen only	1.9	1.9	1.4	0.7	1.9
Percentage showing no naked-eye lesions	29.3	26.8	30.4	30.4	25.3
Percentage showing gross splenic enlargement	3.5	5.8	4.7	4.0	7.3
Percentage showing necrotic areas in liver	19.6	20.1	15.6	28.6	24.9



It will be seen that the percentage of mice coming to necropsy from which we failed to isolate *Bact. aertrycke* is no higher in the groups inoculated with the effective vaccine (*K* and *C*) than in the unvaccinated groups (*H* and *Daily*). Coming to those mice from which *Bact. aertrycke* was isolated, we find that the percentage of mice from which that organism was recovered from the spleen culture *only* is sensibly equal in all groups except *K*, where it is insignificantly smaller. It is certain that, if latent infections were common among mice in the vaccinated groups which had, in fact, died from other causes, the percentage of mice showing this state of affairs would be significantly higher in groups *K* and *C* than in groups *H*, *Daily*, and *J*. The percentages showing no naked-eye lesions confirm this view: there is no significant difference between the vaccinated and unvaccinated. As regards the two lesions most characteristic of enteric infection in the mouse, gross splenic enlargement and necrotic foci in the liver, the vaccinated groups show, if anything, higher frequencies than the unvaccinated. This is in accordance with the increased survival time of the vaccinated mice. Figures which we have obtained from another long-continued epidemic of mouse typhoid show an increase in the frequency of gross splenic enlargement from 1.2 per cent. among mice dying between the sixth and tenth day of exposure to 8.3 per cent. among mice dying on or after the fortieth day, and a corresponding increase from 1.4 to 18.8 per cent. in the frequency of necrotic liver lesions.

#### THE EFFECT OF VACCINATION ON HERD EXPERIENCE.

We now pass to a discussion of the effect of the vaccinations observed in herd experience. First we must ask ourselves, on the basis of individual experimentation and deduction from such experimentation, what advantages we should *expect* the animals treated with the different vaccines to enjoy in comparison with untreated animals when all are exposed alike to the chances and changes of existence in a tainted herd. In the first place we must note that all the vaccinated animals have been subjected to a mortuary selection which has eliminated from 2.56 to 10.40 per cent. of the animals brought under observation. If we suppose that power to resist the toxic products present in the vaccines employed is *some* measure of power to resist the consequences of natural infection in the herd, then we must suppose that the *E* batch of actual entrants to the herd, which consists of a residue left after winnowing the proposed immigrants to the extent of 10.40 per cent., will exhibit more resistance than the *A*'s who had been selected to the extent of only 2.56 per cent. The precise importance of this we cannot determine; we only note the fact.

Leaving this aspect of the problem, which of the vaccinations should we expect to have been efficacious? There seems no *a priori* ground for expecting that the *A* vaccine will improve the prospect of immigrants, apart from some possible non-specific effect. The *B* vaccine is in much the same category, since all the available evidence suggests that the cosmopolitan rough antigen of the

enteric group is valueless as an immunising agent. For the same reason we should expect the *D* vaccine to have little value, with the reservation that it is impossible, by any laboratory test, to be certain that a rough variant of any particular strain is *entirely* free from any trace of the O antigen characteristic of the strain from which it was derived. The properties of the *J* vaccine have been described in some detail above; in the light of the results recorded we should clearly not expect that it would provide any significant protection. On the assumption that the O somatic antigen is the important factor in immunisation, we should expect the remaining vaccines, *C*, *E*, *F*, *G* and *K*, to be of approximately equal value, noting that, if the antigenic value of a vaccine decreases rapidly with storage, we should expect vaccine *K* to be superior to vaccine *C*, over the last period of the experiment.

In Table VI (see Appendix, pp. 279–289) we have given the life tables of the various batches, in full detail for the first 30 days, thereafter at intervals of 5 or 10 days. This is comparable with our earlier tables. But it may be objected that, while the tables for control mice cover the whole period of the experiment, the various batches of vaccinated mice (except *C*) were added over different periods, so that their life tables are not strictly comparable either *inter se* or with the experience of the controls. To test this point Table VII was prepared which gives the probabilities of surviving five days for the *H* and Added Daily mice in periods strictly comparable with the periods of exposure of the vaccinated mice. For exposures of 35 days or more the variations from column to column are large, but for these exposures the numbers of animals concerned are very small. At the earlier ages the variations are small and inconsistent in sign. It seems, therefore, best to use as controls the whole experience of *H* and Added Daily mice<sup>1</sup>.

Table VII.

## ALL DEATHS.

Cage age in days	Comparable with A–G		Comparable with J		Comparable with K	
	<i>H</i>	Added daily	<i>H</i>	Added daily	<i>H</i>	Added daily
0	·9710	·9816	1·000	1·000	·9952	·9932
5	·9635	·9609	·9900	·9762	·9641	·9817
10	·9207	·9121	·8889	·9366	·9206	·8884
15	·7900	·8000	·8011	·8490	·8005	·8508
20	·6919	·6317	·7021	·7178	·7071	·6923
25	·4795	·5795	·5051	·4786	·5238	·5378
30	·5286	·6037	·5600	·5536	·4638	·5124
35	·6486	·6263	·6071	·8387	·5882	·6129
40	·6667	·6290	·6471	·8077	·6333	·6316
45	·7500	·6667	·5455	·8091	·4737	·4500
50	·5833	·8846	·6667	·9412	·8889	·7778
55	·5714	·7826	1·000	·9375	·8750	1·000

With regard to the general course of mortality we find the expected increase to a maximum of mortality in the region of the thirtieth day followed by a

<sup>1</sup> Tables VI A–D (see Appendix, pp. 286–289) provide data for exact comparison should other investigators desire to pursue the subject further.

recovery towards an asymptotic level. There is no doubt that this is the normal picture of herd mortality under our experimental conditions.

We must now decide what criterion of success is the most suitable. The most obvious test of success is naturally to ascertain whether the supposedly immunised members of a community did actually live longer than the controls. As in these experiments virtually all the animals under observation had died before the statistical analysis was made, this merely involves the comparison of the average lengths of life of the different batches with their probable errors. In Table VII A these are shown. Except that *K* has been less successful than *a priori* considerations would suggest, the results are not discrepant with our anticipations. As we have noted before, however, utilising all the data has the disadvantage that a few mice who lived very long might play too prominent a part in determining the general average. In the fourth column of the table the comparison is limited to the first 60 days of observation. By the end of 60 days in all batches fewer, usually far fewer, than 10 per cent. of the exposed to risk are still alive. A comparison on this basis, *i.e.* averaging a variable restricted to the range 0-60, improves the relative position of *K* but leads us to the same general result. We think that these simple and direct comparisons establish the proposition that certain of the vaccines have, appreciably and—in the statistical sense—significantly, lengthened the lives of the exposed to risk. Taking the *H* mice as the standard then the *E*, *F* and *G* mice, which differ insensibly among themselves, show an ad-

Table VII A. *Experiment 1.*

EXPECTATION OF LIFE.								
Total deaths				Specific deaths				
Group	Un-limited	Standard error	Limited to 60	Standard error	Un-limited	Standard error	Limited to 60	Standard error
<i>H</i>	26.27	0.567	25.28	.331	27.93	0.595	26.38	.347
<i>A</i>	33.55	2.068	28.51	.682	36.60	2.164	29.86	.714
<i>B</i>	31.67	1.869	27.50	.683	35.43	2.003	29.28	.732
<i>C</i>	34.24	0.956	30.12	.402	38.74	1.028	32.10	.432
<i>D</i>	35.85	2.747	28.14	.734	39.33	2.885	29.44	.771
<i>E</i>	39.07	2.321	32.38	.780	44.16	2.515	34.97	.845
<i>F</i>	41.71	2.942	32.63	.778	48.93	3.194	35.06	.845
<i>G</i>	45.84	3.466	31.99	.803	52.12	3.684	33.71	.853
<i>J</i>	25.75	1.075	24.77	.715	27.30	1.127	25.87	.750
<i>K</i>	32.62	1.235	29.44	.620	35.39	1.300	30.82	.653
Added daily	29.70	0.769	25.86	.274	33.24	0.809	27.07	.288
Period 1. v. 28-27. xi. 28 to compare with <i>A</i> to <i>G</i> .								
<i>H</i>	26.45	1.271	24.90	.606	29.06	1.345	26.26	.641
<i>C</i>	39.08	2.103	32.61	.805	46.29	2.309	35.31	.884
Added daily	27.26	0.980	25.29	.439	30.08	1.040	26.66	.466
Period 4. xii. 28-5. ii. 29 to compare with <i>J</i> .								
<i>H</i>	26.89	1.266	25.58	.705	28.41	1.313	26.25	.731
<i>C</i>	32.26	1.601	29.82	.740	34.29	1.688	31.14	.780
Added daily	34.64	2.679	27.88	.830	38.29	2.773	28.58	.859
Period 19. ii. 29-9. vii. 29 to compare with <i>K</i> .								
<i>H</i>	25.99	0.620	25.51	.481	27.05	0.648	26.48	.502
<i>C</i>	31.64	1.257	28.58	.565	35.01	1.339	30.16	.602
Added daily	28.37	1.072	26.13	.506	30.61	1.131	27.37	.534

vantage of 28 per cent. The *C* mice, over the same period of exposure, show an advantage of the same order. If the mice added daily are the standard, the advantage is not materially less, 25 per cent. But even the most favoured group only enjoyed slightly more than half the possible life span, 60 days, and one of us (Greenwood, 1928) showed that, of a sample of mice reared under specially favourable conditions, none died in the first 50 days, while of mice under rather less exceptional conditions and observed from the age of 6 months, 89 per cent. were still alive after 60 days' exposure. Were these valid controls, it is obvious that our immunised mice had not been brought into approximately the same state as healthy animals, but it was arbitrary to suppose that the controls *were* valid. We therefore carried out a strictly comparable control experiment. Twenty normal mice were placed in a cage of precisely the type of those used in the experiments on 4. x. 29; for 3 days one mouse was added daily, then for 3½ months three mice were added daily. In all 329 mice were used, of which 273 survived at the end of the experiment. Of the 56 deaths, 15 were classed as not examined, 41 were examined and not found to be specifically infected. The expectation of life at entry limited to 60 days was found to be 56.79 days. In other words, taking round numbers, unprotected mice in our infected herd had an expectation of 25 days, mice immunised as efficiently as we were able to immunise them had an expectation of 32 days and mice living under conditions in all respects the same, save non-exposure to the specific risk, had an expectation of 57 days, respectively 42, 53 and 95 per cent. of the maximum possible. We shall, in a moment, discuss the objections to which this comparison is subject, but, to anticipate a little, we may say that the objections do very little to weaken the essential force of the comparison. Most members of the general public and some scientific writers use language which implies that it is reasonable to hope that bacterial vaccines, or similar immunising agents, may be found which will place the vaccinated, *vis-à-vis* with some particular risk, let us say dying of typhoid fever or of scarlet fever, or of smallpox, precisely in the (mythical) position of King Mithradates who could consume the most deadly poisons as table beverages. So far as these persons are concerned, *that* element of the environment, dosage of infective material in food or drink, contact with infectious persons, is simply eliminated. Naturally these conditions have never been realised (with the possible exception of vaccination against small-pox for a limited period after vaccination) in practice, but it has been believed that their realisation is practically possible.

The results we have obtained in this experiment strengthen our suspicion that they are unrealisable. We have at least succeeded in providing conditions of exposure which overcome the protection afforded by two massive doses of a vaccine containing the antigenic factors that should produce an efficient immunising response. These conditions, too, are conditions of natural infection from member to member of the herd, not of some massive artificial inoculation. So far as antibacterial immunity is concerned there seems to be a critical limit

to the response of the organism to immunisation, whether by artificial inoculation or by sublethal natural infection. This has been overlooked because statistically controlled herd experimentation has been neglected.

This direct comparison is subject to the criticism that it is based upon deaths from all causes, and there is no theoretical reason why the vaccinations should have improved the prospects of the vaccinated when confronted with some cause of death other than aertryckial infection; one should compare the mortality experiences with respect to the specific infection alone. The practical importance of this objection is less than might have been supposed. It appears (see Table VIII) that more than half, perhaps as many as 90 per cent. of all the deaths *were* really attributable to the specific infection against which we attempted to immunise our animals, so that the elimination of the non-specific deaths should not make a very important difference. The pure comparison is, however, made in the right-hand half of Table VII A. The sampling errors of the expectations given there are only approximations. When one is dealing with mortality from all causes in a population, all members of which have been observed from entrance to death or to a particular terminus, the expectation of life is the arithmetic mean of the frequency distribution of the ages at death (or the terminal age), and the standard error of the mean is simply the standard deviation of the frequency divided by the square root of the number of deaths and survivors to the terminal age. If, however, we wished to learn what would be the average length of life of members of a herd subject to one only of the causes of mortality which actually operated, we shall not reach the right answer by taking the average age at death of the members of the herd which, under the real conditions, died of this particular disease. They would then form only a part of the number of deaths and, owing to the mortality from other causes, there would be too few survivors to later ages, the average longevity shown would be too small. What we should have to do would be to construct, from the death-rates of the special disease, a new, fictitious life table setting out how the population would have died had only this cause been operating. The  $d_x$  column of this table will *not* be the deaths at ages from the specific cause of the original table but those deaths respectively multiplied by factors which increase with  $x$ . When we took all deaths,

Table VIII.

Group	Proportions of deaths certainly due to <i>Bact. aertrycke</i>	Proportions of deaths probably due to <i>Bact. aertrycke</i>
<i>A</i>	64.8	91.3
<i>B</i>	62.3	87.1
<i>C</i>	65.2	86.4
<i>D</i>	58.7	90.6
<i>E</i>	61.9	85.2
<i>F</i>	63.2	84.8
<i>G</i>	61.5	88.7
<i>H</i>	64.6	90.9
<i>J</i>	67.5	91.0
<i>K</i>	66.9	90.2
Added daily	64.5	90.4

the expectation of life was an unweighted average; now we have a weighted average with weights substantially correlated with the variables. The standard error of such an average is difficult to calculate and is evidently larger, it might be *many* times larger, than that of the unweighted average. We know of no arithmetically simple device for approximating to the value. In our particular case the difficulty is more apparent than real, because so large a proportion of the deaths was really due to the specific factor that the difference between the actual mean age at death and the correctly determined expectation of life, although distinct enough, is not very large. We have computed a standard error by using as standard deviation that obtained from the all causes data and as  $n$  the number of animals in each group which did actually die of the specific cause, but, for the reasons just given, this is *certainly* an underestimate and the inexactitude increases as the proportion of specific deaths decreases. The comparison appears in the right-hand half of Table VII A. Confining ourselves as before to the limited expectation, it will be seen that this comparison does not modify the conclusions to be drawn from that of the total mortality. Vaccination confers a substantial benefit, but altogether fails to produce a solid immunity.

Having reached this very important conclusion, we must seek to understand the nature of the limitation of the benefit conferred.

We proved in earlier researches that exposure to natural infection within a herd leads to the production of an immunity which, like that under notice, is substantial but far from perfect. We have given reasons for thinking that this immunisation, rather than mortuary selection, explains the decreasing rate of mortality with increasing cage age. It is tempting to suppose that the effect of vaccination would be to place immigrants at entrance into the same position as elder survivors of natural exposure, so that, for instance, if we were comparing death rates, we ought to find that  $q'_x = q_{x+s}$ , where  $q'_x$  is the probability of surviving a day from age  $x$  of a vaccinated animal, while  $q_x$  is the survivorship function for the unvaccinated animals and  $s$  is a constant. An examination of Tables IX and X, which give the probability of surviving

Table IX. *Experiment 1. Probability of survivorship.*

Cage age in days	ALL DEATHS.										Added daily
	A	B	C	D	E	F	G	J	K	H	
5	.990	.990	.996	.987	.987	.984	.994	.995	.993	.988	.987
10	.980	.974	.975	.980	.984	.993	.987	.970	.988	.966	.966
15	.947	.923	.949	.923	.944	.960	.967	.902	.922	.912	.901
20	.863	.822	.887	.852	.937	.911	.881	.759	.847	.800	.811
25	.724	.727	.810	.703	.793	.845	.779	.682	.783	.702	.660
30	.629	.648	.624	.560	.711	.688	.662	.489	.690	.510	.545
35	.554	.551	.643	.720	.693	.623	.692	.568	.621	.502	.592
40	.629	.712	.664	.687	.788	.792	.772	.520	.694	.613	.690
45	.795	.690	.635	.739	.683	.750	.817	.538	.693	.644	.759
50	.806	.621	.806	.794	.750	.825	.828	.857	.634	.574	.745
55	.760	.889	.828	.926	.762	.809	.875	.833	.879	.704	.880
60	.895	.875	.903	.920	.844	.789	.905	1.0	.862	.789	.884

Table IX A. *Experiment 1. Probability of survivorship from life tables.*

Cage age in days	SPECIFIC DEATHS.										Added daily
	A	B	C	D	E	F	G	J	K	H	
5	1.0	.9968	.9979	.9935	1.0	.9935	.9968	1.0	1.0	.9937	.9919
10	.9902	.9772	.9926	.9804	.9902	1.0	.9935	.9798	.9904	.9732	.9706
15	.9533	.9263	.9624	.9300	.9634	.9669	.9735	.9166	.9319	.9226	.9113
20	.8795	.8591	.9068	.8716	.9574	.9241	.9237	.7898	.8643	.8260	.8307
25	.7403	.7668	.8334	.7339	.8289	.8634	.7859	.6874	.7992	.7164	.6781
30	.6413	.6800	.6508	.5889	.7408	.7341	.6744	.6050	.7101	.5423	.5825
35	.5625	.5984	.6629	.7302	.7172	.6734	.7254	.5878	.6434	.5172	.6226
40	.6531	.7270	.6982	.6866	.7981	.8210	.7822	.5448	.7091	.6264	.7088
45	.8197	.6905	.6542	.7559	.6951	.7738	.8169	.5385	.6933	.6438	.7686
50	.8375	.6552	.8230	.7941	.7842	.8246	.8276	.8571	.6698	.5936	.7508
55	.7600	.8889	.8276	.9259	.7857	.8278	.8750	.8333	.9063	.7037	.8796
60	.8947	.8750	.9292	.9200	.8710	.8373	.9274	1.0	.8621	.7895	.8937

through the next 5 days at intervals of 5 days for our observations down to day 60<sup>1</sup>, shows that no mere shifting vertically of the columns would produce agreement. If, for instance, we shift the entries under *F* downwards we shall not find that any such movement will make them agree with *H*<sup>2</sup>. Comparison shows that, at first, all the probabilities are approximately equal, that then those of the vaccinated groups diverge more and more from the controls to a maximum difference round about 30, *i.e.* the probability of surviving from day 25 to day 30, and thereafter the probabilities of survivorship of the vaccinated groups again approximate to those of the controls. For the purpose of studying the matter more closely Table X has been constructed. The vaccinated groups *E*, *F*, *G* (on the whole the most successful groups) are confronted with the weighted average of the controls (the added daily group being twice as large as the *H* group its probabilities have been given twice the weight of the

Table X. *Probability of surviving 5 days.*

Cage age in days	SPECIFIC DEATHS.			Column 2	Graduation of col. 5
	<i>E, F, G</i>	Controls	Difference	Column 3	
0	.9968	.9925	.0043	1.004	1.001
5	.9946	.9715	.0231	1.024	1.009
10	.9679	.9151	.0528	1.058	1.039
15	.9351	.8291	.1060	1.128	1.113
20	.8261	.6909	.1352	1.196	1.217
25	.7164	.5691	.1473	1.259	1.274
30	.7053	.5875	.1178	1.201	1.229
35	.8004	.6813	.1191	1.175	1.126
40	.7619	.7270	.0349	1.048	1.046
45	.8121	.6984	.1137	1.163	1.011
50	.8295	.8210	.0085	1.010	1.002
55	.8786	.8590	.0196	1.023	1.000

<sup>1</sup> Thus the entry .9968 against cage age 0 means that 99.68 per cent. of the *B* animals survived 5 days from entrance. The next entry .9772 means that 97.72 per cent. of the animals which survived 5 days survived a further 5 and so on.

<sup>2</sup> It must be noted that the fact that a simple translation does not produce agreement does not prove that the hypothesis suggested above is false, since, during the first days of exposure risk of death is relatively small for *all* mice. All that is demonstrated is that the simplest form of that hypothesis is inexact.

*H* group). After day 40 the differences are irregular, but they suggest a rise to a single maximum and then a decline. Taking now the ratios of the survivorship probabilities of the vaccinated to the unvaccinated group, we have the figures of the fifth column. The suggestion they convey is that the relative advantage of the vaccinated increases to a maximum at somewhere about the epoch in herd life when the specific mortality rate itself is at a maximum. It looks as if vaccination gave the vaccinated an extra reserve of resistance, which responded to the increasing demands made upon it up to a point and then wore away. Is this capable of quantitative appraisal? A fundamental difficulty is that, owing to the rapid decrement of the exposed to risk, the standard errors of such ratios increase very fast. In fact calculation shows that, for day 45 onwards, the standard errors of the ratios are of the same order of magnitude as the differences of these ratios from unity. They are therefore wholly unreliable as data for the determination of the "law" of change of the ratio. A long shot can, however, be made in the following way. Let us suppose that the ratio starts at unity—no difference between vaccinated and unvaccinated—in respect of mortality in the first 5 days of herd life—and gradually approaches unity again in symmetrical fashion. Then we might suppose that the differences of the several ratios from unity should be approximately represented by areas of a normal curve. As, however, the limit of this curve towards increasing  $x$  is ill-determined, any approximation to its constants must be imperfect. A first attempt was made by the plan of assuming that the first few values formed the tail of a normal curve and deducing the constants of the whole curve from this stump by a method devised by Pearson. The graduations so obtained were not, however, satisfactory. They greatly exaggerated the advantage of the vaccinated at a part of the course where the observed values depend upon reasonably large numbers and are, therefore, fairly trustworthy. A second attempt was then made by directly fitting a normal curve to the seven values from 0.058 to 0.048 inclusive. This, it will be seen, leads to a much better result. Only where the observations are scanty is the divergence gross. Having regard to the necessarily crude method of analysis from a biometric point of view and to the biological, or epidemiological, probability that any "law" *must* be blurred in operation by extraneous factors, we seem justified in saying that a feasible explanation of our results is that suggested. An analogy may help to make the point clear. Suppose that a mouse of a particular category were being bombarded with lethal missiles and his chance of not being hit by any one missile were  $q$  ( $p + q = 1$ ), then if  $n$  bullets were aimed at him, his chance of escaping would be  $q^n$ . For a mouse of different category the chance might be  $q'$ , and under the given circumstances his chance of escape would be  $q'^n$ . Let  $q'$  be greater than  $q$ . Then the ratios of survivorship will be  $(q/q')^n$  which will decrease with  $n$  without limit. If, however,  $q$  and  $q'$  both differ very little from unity, the limit as  $n$  increases will not be zero but  $e^{-k}$ , where  $k$  is a constant and  $e$  the base of the natural logarithms, while, when  $n$  is small, the ratio will differ



little from unity. Now, if we supposed that in successive intervals of 5 days the value of  $n$  varied, was first small, then increased and then again decreased, we should find the ratios of survivorship first near unity, then increasing to some limit and then again falling towards unity. We should have a result having some resemblance to what we find. For reasons set out at length in our last paper, we are satisfied that this scheme is far too simple to be adequate, but it is sufficient to illustrate the general line of argument.

At this point it will be convenient to correct an error published in our last paper. In that paper (Greenwood, Newbold, Topley and Wilson, 1930, p. 257) we printed two tables (Tables VIII and IX) from which we drew the conclusion that the asymptoting of  $q_x$ , which characterises all our experiments, including the present one, was attributable to an insensitiveness of the inhabitants to variations of the environment *after* the first few days of their residence. Table IX, which was a direct comparison of survivorships under contrasting conditions, is not, we think, open to objection and, so far as it goes, sustains the conclusion drawn. But Table VIII is logically invalid. The correlations contained in it were not based as, we think, they should have been upon the individual observations, but upon the *means* of small groups. No doubt if the batches used in each calculation had been of the same size, the coefficients of correlation would have been comparable although, even then, we do not think the method would have been satisfactory. But actually the batches decreased in size. The methodological error involved is this. Suppose we start with two variables  $x$  and  $y$ , in our case  $x$  is the prevailing death-rate in the cage, and  $y$  the length of time a mouse exposed to that death-rate survived after the date to which the death-rate refers. There will be a certain degree of correlation between  $x$  and  $y$ . Now suppose we take the table showing for each value of  $x$  the various associated values of  $y$ , i.e. the arrays of  $y$  for each value of  $x$ , and form a new variable  $y'$  by dividing up at random the  $n_x$  values of  $y$  corresponding to the particular  $x$  into, say,  $k$  sets and taking the  $k$  means as our new variable  $y'$ , the correlation of  $x$  with  $y'$  will be numerically larger than the correlation of  $x$  with  $y$ . This is easy to demonstrate algebraically, but it will be sufficient to note that if the array is reduced to a single value by taking the mean of the array as variate, then, if the regression be linear and  $x$  and  $y$  are correlated, the correlation of  $x$  with  $y'$  must be perfect. Hence the decreasing absolute values of the coefficients of correlation in the table might merely be expressions of the fact that as the batches used decreased in size one was approaching the value of the proper variate correlation of  $x$  and  $y$ ,  $y'$  was becoming the same as  $y$ .

An obvious check was to compare the *regression* coefficients, which would not be affected by the procedure and we found that these did not, having regard to errors of sampling, show any significant or regular decrement. We think therefore that the support Table VIII of the last paper appeared to give to the conclusion was illusory. Using the material of the present experiment we have studied the proper measure, viz. the correlation of prevailing death-

rate and individual survivorship and found it both for vaccinated and control mice to be insignificant. We have also prepared a table (Table XI) comparable in form with Table IX of the previous paper. This does not confirm the suggestion of that table. It must be noted, however, that the contrasting levels of mortality are not the same as before. The position is that our previous surmise may be correct, but that the evidence we tendered before and the material we have derived from this experiment do not justify it. Biologically, the surmise still commends itself to us as a reasonable one, but it is *not*, as we suggested, statistically established.

Table XI. *Cage 1.*

Cage age <i>x</i>	Mean length of after life from day <i>x</i>		Difference	Numbers of groups	
	Low death-rate (under .025) just before day <i>x</i>	High death-rate (over .035) just before day <i>x</i>		Low death-rate	High death-rate
	Group <i>C</i>				
0	43.37	32.60	10.77	13	16
5	34.37	26.33	8.04	26	6
10	30.11	26.31	3.80	11	13
15	31.92	21.59	10.33	12	16
20	22.82	15.95	6.87	18	11
25	22.32	8.85	13.47	16	6
30	27.32	12.05	15.27	6	19
35	48.27	16.94	31.33	8	19
40	42.97	32.33	10.64	18	10
Group <i>H</i>					
0	25.35	27.20	- 1.85	13	16
5	22.48	20.35	2.13	26	6
10	16.20	19.21	- 3.01	11	13
15	13.37	15.26	- 1.89	12	16
20	11.33	9.82	1.51	18	11
25	11.88	10.23	1.65	16	6
30	9.25	15.08	5.83	6	19
35	29.43	9.05	20.38	7	16
40	32.81	14.73	18.08	17	6

From the results obtained in the present experiment we conclude that the protection afforded by vaccination is neither absolute, nor independent of subsequent happenings in the infected herd. It increases the average resistance, *vis-à-vis* with the particular risk concerned, and in so doing it increases the probability that the first reception of a dose of infective material will result in a latent infection associated with a further immunising response; but the value accruing from the artificial immunisation will depend in large part on the magnitude of the doses of bacilli subsequently received, and on the intervals between these doses. If the bombardment to which a mouse is subjected is intense and continuous, the relative increase in its resistance will be of little avail.

Incidentally, this way of thinking of artificial immunisation enables us to think of such vaccination as a communal measure in a more sensible way. Suppose the members of our herds had been dispersed after some 30-40 days of communal existence, farmed out, perhaps in little groups of twos and threes. At this time the number of surviving vaccinated animals would (assuming

equal numbers of entrants of the two groups) be more than double the number of surviving controls. Under the new conditions a good many would die of the acquired infection but, in comparison with the mortality rate of the herd, very few. The advantage conferred by vaccination would *approximate* to a saving of 50 per cent. of deaths. The value of the immunisation when a herd is exposed to temporary but intensely unfavourable conditions is enormous. But, if the herd conditions persist, although the vaccinated animals have an advantage it is not, from the point of view of the community, a very great one. If we intend to expose a herd—whether troops on active service or hospital attendants—to transitory but intense risks of infection, a method of vaccination may be expected to render good service. The vaccination of 100 per cent. of a community steadily, from generation to generation, exposed to risk of infection, will no more eliminate the disease than, as we showed in an earlier paper, will the exclusion of infected immigrants to a community, in which the disease has existed and still smoulders, lead to elimination.

We have now discussed the broad results of this experiment, so far as concerns the effect of immunisation with the most efficient of the vaccines employed. The subsidiary question of the relative efficiency of the eight different vaccines employed may be dealt with very briefly. Table VII A sets out the relevant data.

The results show clearly that those vaccines which contain the smooth O antigen (*C*, *E*, *F*, *G* and *K*) are all effective, in the sense outlined above, and that their efficiency differs insignificantly *inter se* when comparisons are made over the same period of time. It is clearly immaterial whether the H components are present (vaccines *C*, *E* and *K*), incompletely represented (vaccine *F*) or altogether absent (vaccine *G*). Nor is a killed broth culture (vaccine *E*), which might contain bacterial products not present in the bacterial suspensions, superior to the saline suspensions of bacilli (vaccines *C*, *F* or *G*). The inefficacy of the H components is confirmed by results obtained with vaccine *D*, containing the H, but not the O, antigens of *Bact. aertrycke*. There is a suggestion that a minor degree of protection may result from entirely non-specific immunisation. The *A* mice, vaccinated with a staphylococcal suspension, and the *B* mice, vaccinated with a rough strain of *Bact. typhosum*, show a slight advantage over the H, and the Added Daily mice. It will be noted that the *D* mice fall into the same category. The *J* mice, vaccinated with *Past. pseudotuberculosis* show no advantage; their expectation of life is less than that of any other group. For this we can offer no explanation.

There is a suggestion, falling far short of demonstration, that the efficacy of the *C* vaccine diminished slightly on storage. Taking the three periods, 1. v. 28 to 27. xi. 28, 4. xii. 28 to 5. ii. 29, and 19. ii. 29 to 9. vii. 29, it will be noted that the relative advantage of the *C* over the *H* mice showed a progressive diminution; during the last period the freshly prepared vaccine *K* gave slightly better results than vaccine *C*, which had at that time been stored for slightly more than a year.

## CONCLUSIONS.

*(a) With regard to the effectiveness of different vaccines.*

1. The efficacy of a *Bact. aertrycke* vaccine, as an immunising agent, appears to be determined by its content in the O somatic antigen. We have obtained no indication that other components have any significant effect.

2. There is a suggestion that the effectiveness of a vaccine containing the O antigen may diminish slightly on storage; but such loss is very slight over a period of 1 year.

3. There is a suggestion that the injection of bacteria unrelated to *Bact. aertrycke* may result in a minor increase in resistance to infection with that organism; but such increase is trivial in comparison with that which follows the injection of the specific O antigen, and is not obtained with all non-specific agents.

*(b) With regard to the influence of efficient vaccination on herd infection.*

4. Vaccinated mice show a significant advantage as compared with unvaccinated mice in respect of their expectation of life, unlimited or limited to 60 days.

5. This advantage is not uniform over the whole period of exposure. As judged by the chance of survival over the succeeding 5 days, it is not apparent on the day of entry, or during the first week of exposure, probably because the risk of dying of a fatal infection within the first 10 days or so of residence in the cage is in any case slight. The advantage of the vaccinated mice, on this test, increases to a maximum between the twenty-fifth and thirtieth days of cage life and thereafter declines, so that a vaccinated mouse which has survived exposure for a considerable period (50–60 days in this experiment) enjoys no significant advantage over an unvaccinated mouse which has survived for the same period.

6. The protection afforded is in no sense absolute. Judged from the experience in this herd of mice it might be regarded as of little account, in that it has little if any influence on the ultimate mortality from the specific disease. Our results offer no suggestion that antibacterial immunisation, with the most efficient available vaccine, will afford protection against severe and prolonged exposure to infection, or will, under such conditions, ameliorate the course of events in an infected herd. The vaccinated mice, in the presence of infection, are never placed in the same position as normal mice in a non-infected herd. This conclusion does *not* involve the deduction that vaccination is useless. Under other conditions of exposure—especially where the risk of infection is limited in time—the advantage afforded by vaccination might well make the difference between effective immunity and death.

We should wish to express our indebtedness to the Medical Research Council, who have defrayed the expenses of this work, and have provided the whole-time services of one of us (J. W.).

## REFERENCES.

- ARKWRIGHT, J. A. (1927). *J. Path. and Bact.* **30**, 345.  
BRÜCKNER, G. (1911). *Zeitschr. f. Immunitäts.* **8**, 439.  
FELIX, A. (1924). *J. Immunol.* **9**, 115.  
GREENWOOD, M. (1928). *J. Hygiene*, **28**, 267.  
GREENWOOD, M., NEWBOLD, E. M., TOPLEY, W. W. C. and WILSON, J. (1930). *Ibid.* **30**, 240.  
IBRAHIM, H. M. and SCHÜTZE, H. (1928). *Brit. J. Exp. Path.* **9**, 353.  
LANGE, B. and YOSHIOKA, M. (1924). *Zeitschr. f. Hyg.* **101**, 451.  
LOEFFLER, F. (1906). *Leuthold Gedänschrift*, **1**, 247.  
LYNCH, C. (1922). *J. Exp. Med.* **36**, 15.  
NEUFELD, F. (1924). *Zeitschr. f. Hyg.* **101**, 466.  
ORNSTEIN, O. (1922). *Ibid.* **96**, 48.  
ROBERTSON, M. and FELIX, A. (1930). *Brit. J. Exp. Path.* **11**, 14.  
SCHÜTZE, H. (1928). *Arch. f. Hyg.* **100**, 181.  
—— (1930). *Brit. J. Exp. Path.* **11**, 34.  
TOPLEY, W. W. C. (1926). *Lancet*, **i**, 645.  
—— (1929). *Ibid.*, **i**, 1337.  
TOPLEY, W. W. C. and WILSON, G. S. (1923). *J. Hygiene*, **21**, 237.  
TOPLEY, W. W. C., WILSON, J. and LEWIS, E. R. (1925). *Ibid.* **23**, 421.  
WEBSTER, L. T. (1922). *J. Exp. Med.* **36**, 71.  
WOLF, K. (1908). *München. med. Wochenschr.* **55**, 270.  
YOSHIDA, E. (1909). *Arch. f. Hyg.* **69**, 21.

## APPENDIX

Table VI.

Cage age in days	C mice				D mice			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000.00	10.37	.0010373	38.74	10000.00	—	—	39.33
1	9989.63	—	—	37.78	10000.00	64.52	.0064516	38.33
2	9989.63	—	—	36.78	9935.48	—	—	37.57
3	9989.63	10.40	.0010406	35.78	9935.48	—	—	36.57
4	9979.23	—	—	34.81	9935.48	—	—	35.57
5	9979.23	—	—	33.81	9935.48	—	—	34.57
6	9979.23	—	—	32.81	9935.48	32.47	.0032680	33.57
7	9979.23	20.99	.0021031	31.81	9903.01	64.94	.0065574	32.68
8	9958.24	21.01	.0021097	30.88	9838.08	64.94	.0066007	31.89
9	9937.24	31.61	.0031813	29.94	9773.14	32.47	.0033223	31.10
10	9905.62	63.50	.0064103	29.04	9740.67	97.41	.0100000	30.20
11	9842.12	74.16	.0075350	28.22	9643.26	129.88	.0134680	29.50
12	9767.96	53.09	.0054348	27.43	9513.39	97.41	.0102389	28.90
13	9714.88	106.52	.0109649	26.58	9415.98	194.81	.0206897	28.19
14	9608.35	74.98	.0078038	25.87	9221.17	162.34	.0176056	27.78
15	9533.37	128.83	.0135135	25.07	9058.82	98.11	.0108303	27.27
16	9404.54	118.77	.0126292	24.40	8960.71	164.72	.0183824	26.56
17	9285.77	205.87	.0221704	23.71	8795.99	99.58	.0113208	26.05
18	9079.90	151.69	.0167064	23.24	8696.42	265.54	.0305344	25.34
19	8928.21	283.09	.0317073	22.62	8430.88	535.29	.0634921	25.12
20	8645.12	263.30	.0304569	22.35	7895.58	501.84	.0635593	25.79
21	8381.82	374.48	.0446780	22.03	7393.74	436.90	.0590909	26.51
22	8007.33	288.35	.0360111	22.04	6956.84	407.23	.0585366	27.14
23	7718.98	244.69	.0317003	21.84	6549.61	477.58	.0729167	27.80
24	7474.29	269.75	.0360902	21.54	6072.04	277.58	.0457143	28.95
25	7204.54	508.16	.0705329	21.33	5794.46	453.78	.0783132	29.31
26	6696.38	524.76	.0783646	21.91	5340.67	501.81	.0939597	30.76
27	6171.62	643.60	.1042830	22.73	4838.87	509.35	.1052632	32.90
28	5528.03	499.38	.0903361	24.32	4329.51	476.98	.1101695	35.71
29	5028.65	339.93	.0675991	25.69	3852.53	440.29	.1142857	39.07
30	4688.71	353.42	.0753769	26.51	3412.24	110.07	.0322581	43.04
35	3108.26	230.69	.0742188	33.66	2491.50	223.12	.0895522	52.88
40	2170.16	178.72	.0823529	42.19	1710.58	37.19	.0217391	71.11
45	1419.74	26.29	.0185185	58.33	1293.08	76.06	.0588235	88.29
50	1168.51	67.16	.0574713	65.32	1026.86	38.03	.0370370	105.61
55	967.04	—	—	73.52	950.79	—	—	108.86
60	898.53	—	—	73.95	874.73	—	—	113.11
70	744.66	28.10	.0377359	78.24	684.57	—	—	132.78
80	614.72	—	—	83.84	646.54	38.03	.0588235	130.26
90	600.08	15.00	.0250000	75.75	570.48	—	—	137.30
100	540.07	30.00	.0555556	73.50	532.44	—	—	137.00
110	465.06	—	—	74.99	418.35	—	—	162.50
120	420.06	31.12	.0740741	72.25	418.35	—	—	152.50
130	357.83	—	—	—	418.35	—	—	—
140	309.67	—	—	—	418.35	—	—	—
150	277.07	—	—	—	418.35	—	—	—
160	244.48	—	—	—	342.29	—	—	—
170	206.87	—	—	—	342.29	—	—	—
180	150.45	—	—	—	342.29	—	—	—
190	150.45	—	—	—	304.25	—	—	—
200	128.96	—	—	—	266.22	—	—	—
210	128.96	—	—	—	190.16	—	—	—
220	128.96	—	—	—	190.16	—	—	—
230	103.16	—	—	—	190.16	—	—	—
240	—	—	—	—	152.13	—	—	—
270	—	—	—	—	152.13	38.03	.2500000	—
280	—	—	—	—	114.10	—	—	—
320	—	—	—	—	114.10	—	—	—

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Table VI (cont.)

Cage age in days	E mice				F mice			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000-00	—	—	44-16	10000-00	32-26	-0032258	48-93
1	10000-00	—	—	43-16	9967-74	—	—	48-09
2	10000-00	—	—	42-16	9967-74	—	—	47-09
3	10000-00	—	—	41-16	9967-74	—	—	46-09
4	10000-00	—	—	40-16	9967-74	32-57	-0032680	45-09
5	10000-00	—	—	39-16	9935-17	—	—	44-24
6	10000-00	—	—	38-16	9935-17	—	—	43-24
7	10000-00	—	—	37-16	9935-17	—	—	42-24
8	10000-00	32-68	-0032680	36-16	9935-17	—	—	41-24
9	9967-32	65-57	-0065789	35-27	9935-17	—	—	40-24
10	9901-75	—	—	34-50	9935-17	32-79	-0033003	39-24
11	9901-75	32-90	-0033226	33-50	9902-38	32-79	-0033113	38-36
12	9868-85	131-58	-0133333	32-61	9869-59	131-16	-0132890	37-49
13	9737-26	98-69	-0101351	32-05	9738-43	33-01	-0033898	36-99
14	9638-57	99-37	-0103093	31-37	9705-42	99-04	-0102041	36-11
15	9539-21	67-18	-0070423	30-69	9606-39	99-04	-0103093	35-48
16	9472-03	100-77	-0106383	29-91	9507-35	66-02	-0069444	34-84
17	9371-26	33-95	-0036232	29-22	9441-33	231-89	-0245614	34-08
18	9337-31	136-31	-0145985	28-33	9209-44	165-64	-0179856	33-93
19	9201-00	68-16	-0074074	27-74	9043-80	166-86	-0184502	33-54
20	9132-84	206-00	-0225564	26-94	8876-94	234-49	-0264151	33-16
21	8926-84	379-13	-0424710	26-55	8642-45	133-99	-0155039	33-05
22	8547-71	173-73	-0203252	26-71	8508-46	368-48	-0433071	32-56
23	8373-97	527-77	-0630252	26-25	8139-98	201-82	-0247934	33-01
24	7846-20	285-32	-0363636	26-98	7938-17	273-73	-0344828	32-84
25	7560-89	322-50	-0426540	26-98	7664-44	479-03	-0625000	32-99
26	7238-38	716-67	-0990099	27-16	7185-41	523-21	-0728155	34-16
27	6521-71	217-39	-0333333	29-09	6662-20	317-25	-0476190	35-80
28	6304-32	327-97	-0520231	29-07	6344-95	430-17	-0677966	36-57
29	5976-35	368-91	-0617284	29-65	5914-79	288-53	-0487805	38-19
30	5607-44	336-45	-0600000	30-56	5626-26	365-34	-0649351	39-13
35	4021-78	309-37	-0769231	36-63	3788-81	236-80	-0625000	51-83
40	3209-69	117-43	-0365854	40-39	3110-56	163-71	-0526316	57-66
45	2231-13	79-68	-0357143	52-08	2406-98	168-91	-0701754	68-76
50	1749-64	83-32	-0476190	60-74	1984-71	168-91	-0851064	78-03
55	1374-71	—	—	71-60	1642-86	86-47	-0526316	88-93
60	1197-33	44-35	-0370370	76-76	1375-60	45-85	-0333333	100-81
70	886-91	—	—	91-50	1238-04	45-85	-0370370	101-77
80	798-22	—	—	90-83	871-21	—	—	132-94
90	709-53	—	—	91-31	779-51	—	—	137-93
100	665-18	44-35	-0666667	87-17	687-80	—	—	145-66
110	532-15	—	—	97-50	596-09	—	—	157-30
120	443-46	—	—	106-30	550-24	—	—	159-78
130	399-11	—	—	—	550-24	—	—	—
140	354-77	—	—	—	550-24	—	—	—
150	354-77	—	—	—	453-95	—	—	—
160	310-42	—	—	—	453-95	—	—	—
170	266-07	—	—	—	453-95	—	—	—
180	266-07	—	—	—	453-95	—	—	—
190	266-07	—	—	—	403-51	—	—	—
200	221-73	—	—	—	403-51	—	—	—
210	177-38	—	—	—	353-07	—	—	—
220	177-38	—	—	—	353-07	—	—	—
230	177-38	—	—	—	302-63	—	—	—
240	177-38	—	—	—	302-63	—	—	—
250	177-38	—	—	—	302-63	—	—	—
260	177-38	—	—	—	252-19	—	—	—
270	177-38	—	—	—	252-19	—	—	—
280	177-38	—	—	—	189-15	—	—	—
290	133-04	—	—	—	189-15	—	—	—
300	133-04	—	—	—	189-15	—	—	—
310	133-04	—	—	—	189-15	—	—	—
320	—	—	—	—	189-15	—	—	—
330	—	—	—	—	189-15	—	—	—
340	—	—	—	—	189-15	—	—	—
350	—	—	—	—	189-15	—	—	—
360	—	—	—	—	189-15	63-05	-3333333	—
370	—	—	—	—	126-10	—	—	—
380	—	—	—	—	126-10	—	—	—
390	—	—	—	—	126-10	—	—	—

Table VI (*cont.*)

## SPECIFIC DEATHS.

Expectation of life limited to 60 days ahead.

Cage age in days	A	B	C	D	E	F	G	H	J	K	Added daily
0	29.86	29.28	32.10	29.44	34.97	35.06	33.71	26.38	25.87	30.82	27.07
1	28.92	28.34	31.22	28.52	34.09	34.31	32.86	25.43	24.90	29.89	26.16
2	27.99	27.41	30.31	27.79	33.20	33.45	32.00	24.45	23.93	28.97	25.26
3	27.06	26.56	29.39	26.87	32.31	32.58	31.14	23.51	22.96	28.04	24.37
4	26.13	25.62	28.50	25.96	31.42	31.70	30.38	22.53	21.99	27.12	23.47
5	25.19	24.68	27.58	25.14	30.53	30.94	29.52	21.63	21.02	26.19	22.55
6	24.26	23.74	26.67	24.22	29.64	30.06	28.65	20.67	20.05	25.27	21.64
7	23.33	23.02	25.74	23.28	28.75	29.18	27.79	19.73	19.08	24.34	20.79
8	22.47	22.16	24.88	22.50	27.86	28.31	26.92	18.83	18.40	23.47	19.93
9	21.61	21.36	24.00	21.73	27.04	27.43	26.15	18.04	17.43	22.60	19.23
10	20.74	20.49	23.16	20.87	26.30	26.56	25.37	17.22	16.55	21.77	18.39
11	19.94	19.75	22.37	20.14	25.39	25.76	24.50	16.36	15.83	21.05	17.66
12	19.14	19.20	21.61	19.49	24.57	24.97	23.87	15.63	15.11	20.49	16.95
13	18.39	18.33	20.80	18.75	23.99	24.41	23.07	15.03	14.38	19.81	16.31
14	17.83	17.70	20.10	18.22	23.32	23.60	22.28	14.33	13.71	19.28	15.64
15	16.97	17.20	19.32	17.61	22.65	22.96	21.63	13.52	12.97	18.53	15.15
16	16.44	16.50	18.65	16.87	21.90	22.29	21.21	12.98	12.29	17.70	14.64
17	15.74	16.11	17.95	16.25	21.22	21.54	20.85	12.35	11.75	17.43	14.16
18	15.03	15.42	17.42	15.51	20.39	21.16	20.06	11.59	10.92	16.63	13.70
19	14.89	15.10	16.78	15.05	19.77	20.64	19.34	11.10	10.80	16.36	13.38
20	14.25	14.86	16.39	15.12	19.00	20.11	18.89	10.87	10.74	16.31	13.00
21	13.77	14.59	15.95	15.27	18.52	19.75	18.84	10.59	10.52	16.04	12.74
22	13.42	14.41	15.76	15.28	18.41	19.15	18.52	10.05	10.06	15.97	12.70
23	13.41	14.44	15.40	15.30	17.88	19.10	18.78	9.77	9.89	15.55	12.80
24	13.63	14.22	14.97	15.57	18.15	18.69	19.06	9.67	9.99	15.35	12.94
25	13.66	14.05	14.60	15.30	17.92	18.44	19.14	9.31	9.66	15.19	13.34
26	13.86	14.10	14.76	15.67	17.81	18.75	19.18	9.30	9.98	15.47	13.79
27	14.02	14.78	15.07	16.36	18.83	19.31	20.18	9.73	10.72	15.68	14.49
28	14.50	14.43	15.88	17.36	18.57	19.38	21.51	9.94	11.18	15.75	15.13
29	14.84	14.84	16.53	18.59	18.69	19.41	22.17	10.28	11.98	15.91	15.79
30	15.47	15.15	16.82	20.09	19.01	19.53	23.36	10.45	12.35	15.98	16.90
35	21.55	20.02	19.98	22.53	21.39	24.45	27.87	12.98	15.04	19.19	21.83
40	28.06	22.73	23.90	28.47	22.32	25.45	32.12	15.05	21.45	22.28	26.36
45	30.39	28.69	32.11	33.89	27.49	28.52	36.55	18.02	34.45	27.32	30.46
50	32.46	39.58	35.47	39.41	31.09	30.79	41.82	25.27	37.33	36.22	36.92
55	39.23	41.49	39.85	39.56	35.70	33.67	45.63	31.41	41.56	36.42	38.96
60	40.97	45.02	40.17	40.18	37.39	36.90	47.54	36.47	39.23	38.83	40.77



Table VI (cont.)

Cage age in days	J mice				K mice			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000-00	—	—	27-30	10000-00	—	—	35-39
1	10000-00	—	—	26-30	10000-00	—	—	34-39
2	10000-00	—	—	25-30	10000-00	—	—	33-39
3	10000-00	—	—	24-30	10000-00	—	—	32-39
4	10000-00	—	—	23-30	10000-00	—	—	31-39
5	10000-00	—	—	22-30	10000-00	—	—	30-39
6	10000-00	—	—	21-30	10000-00	—	—	29-39
7	10000-00	151-52	·0151515	20-30	10000-00	24-04	·0024038	28-39
8	9848-49	—	—	19-61	9975-96	24-04	·0024096	27-45
9	9848-49	50-51	·0051282	18-61	9951-92	48-08	·0048309	26-52
10	9797-98	152-30	·0155440	17-70	9903-85	96-15	·0097087	25-64
11	9645-68	152-30	·0157895	16-97	9807-69	168-68	·0171990	24-89
12	9493-38	152-30	·0160428	16-24	9639-01	120-49	·0125000	24-32
13	9341-08	205-30	·0219780	15-50	9518-52	192-78	·0202532	23-62
14	9135-78	154-84	·0169492	14-83	9325-74	96-39	·0103359	23-10
15	8980-94	206-46	·0229885	14-08	9229-35	48-58	·0052632	22-33
16	8774-48	309-69	·0352941	13-40	9180-78	340-93	·0371353	21-45
17	8464-79	105-15	·0124224	12-87	8839-85	73-67	·0083333	21-26
18	8359-64	630-92	·0754717	12-03	8766-18	344-74	·0393258	20-43
19	7728-72	635-24	·0821918	11-97	8421-44	444-53	·0527859	20-25
20	7093-48	483-65	·0681818	11-99	7976-91	322-05	·0403727	20-35
21	6609-84	325-07	·0491803	11-84	7654-86	398-95	·0521173	20-18
22	6284-76	487-61	·0775862	11-42	7255-91	224-41	·0309278	20-26
23	5797-15	595-97	·1028037	11-34	7031-50	327-63	·0465950	19-90
24	5201-18	325-07	·0625000	11-58	6703-87	328-87	·0490566	19-84
25	4876-11	595-97	·1222222	11-32	6375-00	480-65	·0753968	19-84
26	4280-14	650-15	·1518987	11-83	5894-34	410-04	·0695652	20-42
27	3629-99	440-00	·1212121	12-86	5484-30	334-72	·0610329	20-91
28	3189-99	447-72	·1403509	13-56	5149-58	336-40	·0653266	21-23
29	2742-28	279-82	·1020408	14-69	4813-18	286-19	·0594595	21-68
30	2462-45	391-75	·1590909	15-31	4526-99	364-24	·0804598	22-02
35	1447-37	173-68	·1200000	19-51	2912-49	296-64	·1018518	27-89
40	788-47	—	—	28-63	2065-15	137-68	·0666667	33-56
45	424-56	60-65	·1428571	45-88	1431-83	27-54	·0192308	42-21
50	363-91	—	—	48-44	959-03	—	—	56-64
55	303-26	—	—	52-23	869-12	29-97	·0344828	57-14
60	303-26	—	—	47-23	749-25	—	—	61-00
70	303-26	—	—	37-23	689-31	—	—	55-57
80	161-74	—	—	55-00	536-30	—	—	60-06
90	161-74	—	—	45-00	405-61	—	—	67-45
100	161-74	—	—	35-00	405-61	—	—	57-45
110	161-74	—	—	25-00	338-01	—	—	57-94
120	161-74	—	—	15-00	270-40	—	—	61-42
130	—	—	—	—	270-40	—	—	—
140	—	—	—	—	202-80	—	—	—
150	—	—	—	—	202-80	—	—	—
160	—	—	—	—	162-24	—	—	—
170	—	—	—	—	162-24	—	—	—
180	—	—	—	—	162-24	—	—	—
190	—	—	—	—	121-68	—	—	—
200	—	—	—	—	—	—	—	—

Table VI (cont.)

Cage age in days	G mice				H mice			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000-00	—	—	52-12	10000-00	10-53	-0010526	27-93
1	10000-00	—	—	51-12	9989-47	—	—	26-96
2	10000-00	—	—	50-12	9989-47	21-07	-0021097	25-96
3	10000-00	32-36	-0032362	49-12	9968-40	—	—	25-01
4	9967-64	—	—	48-27	9968-40	31-71	-0031813	24-01
5	9967-64	—	—	47-27	9936-69	10-58	-0010650	23-09
6	9967-64	—	—	46-27	9926-10	21-25	-0021413	22-11
7	9967-64	—	—	45-27	9904-85	42-56	-0042965	21-16
8	9967-64	32-47	-0032573	44-27	9862-29	106-50	-0107991	20-25
9	9935-17	32-47	-0032680	43-42	9755-79	85-20	-0087336	19-46
10	9902-70	—	—	42-56	9670-59	74-64	-0077178	18-63
11	9902-70	98-05	-0099010	41-56	9595-95	149-27	-0155556	17-77
12	9804-66	32-68	-0033333	40-97	9446-68	234-83	-0248588	17-04
13	9771-97	32-68	-0033445	40-10	9211-85	171-18	-0185830	16-46
14	9739-29	98-71	-0101351	39-24	9040-66	118-39	-0130952	15-77
15	9640-58	197-42	-0204778	38-63	8922-27	291-30	-0326481	14-97
16	9443-16	231-94	-0245614	38-43	8630-98	238-25	-0276035	14-46
17	9211-23	33-62	-0036496	38-38	8392-73	152-40	-0181582	13-85
18	9177-61	67-73	-0073801	37-52	8240-34	351-12	-0426099	13-10
19	9109-88	204-72	-0224719	36-80	7889-22	519-32	-0658263	12-66
20	8905-16	379-68	-0426357	36-63	7369-90	467-58	-0634441	12-52
21	8525-48	242-60	-0284553	37-24	6902-32	289-92	-0420032	12-33
22	8282-89	485-19	-0585774	37-32	6612-40	449-82	-0680272	11-85
23	7797-70	450-53	-0577778	38-61	6162-58	541-77	-0879121	11-68
24	7347-17	348-21	-0473934	39-95	5620-81	340-66	-0606061	11-76
25	6998-96	313-39	-0447761	40-91	5280-16	522-34	-0989247	11-48
26	6685-57	591-95	-0885417	41-80	4757-82	651-91	-1370192	11-69
27	6093-62	595-35	-0977011	44-82	4105-91	466-58	-1136364	12-47
28	5498-27	352-45	-0641026	48-61	3639-33	444-68	-1221865	13-00
29	5145-81	425-86	-0827586	50-91	3194-65	331-30	-1037037	13-74
30	4719-95	283-91	-0601504	54-46	2863-35	241-63	-0843882	14-27
35	3423-72	223-29	-0652174	69-11	1480-82	223-99	-1512605	20-08
40	2677-92	226-30	-0845070	82-76	927-57	63-53	-0684932	25-91
45	2187-60	150-87	-0689656	95-99	597-20	76-24	-1276596	34-16
50	1810-42	—	—	110-66	354-51	26-26	-0740741	51-22
55	1584-12	37-72	-0238095	121-01	249-47	26-26	-1052632	66-53
60	1469-08	38-66	-0263158	125-29	196-95	—	—	78-74
70	1314-44	38-66	-0294118	129-53	157-56	—	—	86-80
80	1117-40	—	—	141-36	144-43	—	—	84-28
90	1117-40	—	—	131-36				
100	1117-40	—	—	121-36				
110	1077-49	39-91	-0370370	115-66				
120	1037-59	—	—	110-08				
130	954-58	41-50	-0434783	—				
140	871-57	41-50	-0476190	—				
150	788-57	—	—	—				
160	700-95	—	—	—				
170	657-14	—	—	—				
180	657-14	—	—	—				
190	613-33	43-81	-0714286	—				
200	525-71	—	—	—				
210	438-09	—	—	—				
220	394-28	—	—	—				
230	350-47	—	—	—				
240	300-41	—	—	—				
250	300-41	—	—	—				
260	300-41	—	—	—				
270	250-34	—	—	—				
280	200-27	—	—	—				
290	150-20	—	—	—				
300	150-20	—	—	—				
360	150-20	—	—	—				
450	100-14	—	—	—				

Table VI (cont.)

## SPECIFIC DEATHS.

## Added Daily mice

Cage age in days	$l_x$	$d_x$	$q_x$	$e_x$
0	10000.00	16.08	.0016077	33.24
1	9983.92	10.73	.0010747	32.29
2	9973.19	21.51	.0021563	31.33
3	9951.69	21.52	.0021622	30.40
4	9930.17	10.78	.0010852	29.46
5	9919.39	16.16	.0016295	28.49
6	9903.23	48.52	.0048993	27.54
7	9854.71	37.80	.0038356	26.67
8	9816.91	129.67	.0132086	25.78
9	9687.25	59.46	.0061384	25.11
10	9627.78	113.65	.0118044	24.28
11	9514.13	135.53	.0142450	23.55
12	9378.60	179.00	.0190862	22.88
13	9199.60	157.96	.0171699	22.31
14	9041.64	267.70	.0296073	21.69
15	8773.95	251.94	.0287141	21.34
16	8522.01	269.58	.0316333	20.96
17	8252.43	286.85	.0347594	20.63
18	7965.58	359.56	.0451389	20.35
19	7606.02	317.38	.0417277	20.29
20	7288.64	375.65	.0515385	20.15
21	6913.00	473.26	.0684597	20.22
22	6439.73	501.87	.0779334	20.67
23	5937.86	469.82	.0791230	21.37
24	5468.04	525.66	.0961337	22.17
25	4942.38	472.35	.0955711	23.47
26	4470.03	480.59	.1075130	24.90
27	3989.44	396.03	.0992701	26.84
28	3593.41	341.67	.0950820	28.74
29	3251.74	372.66	.1146026	30.71
30	2879.08	276.83	.0961538	33.61
35	1792.52	135.89	.0758123	47.63
40	1270.55	99.78	.0785340	61.37
45	976.53	53.88	.0551724	74.24
50	733.18	6.79	.0092593	93.05
55	644.91	6.79	.0105263	100.41
60	576.37	13.72	.0238095	106.98
70	455.86.	7.24	.0158730	123.94
80	419.32	—	—	124.32
90	382.53	7.36	.0192308	125.79
100	353.11	—	—	125.98
110	308.97	—	—	133.19
120	293.90	—	—	129.84
130	270.70	—	—	—
140	270.70	—	—	—
150	238.10	—	—	—
160	221.68	—	—	—
170	196.02	8.91	.0454545	—
180	169.29	—	—	—
190	160.38	—	—	—
200	151.47	—	—	—
210	151.47	—	—	—
220	151.47	—	—	—
230	142.56	—	—	—
240	142.56	—	—	—
250	133.65	—	—	—
260	133.65	—	—	—
270	124.11	—	—	—
280	114.56	—	—	—

Table VI (cont.)

Cage age in days	A mice				B mice			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000-00	—	—	36-60	10000-00	—	—	35-43
1	10000-00	—	—	35-60	10000-00	—	—	34-43
2	10000-00	—	—	34-60	10000-00	32-26	·0032258	33-43
3	10000-00	—	—	33-60	9967-74	—	—	32-54
4	10000-00	—	—	32-60	9967-74	—	—	31-54
5	10000-00	—	—	31-60	9967-74	—	—	30-54
6	10000-00	—	—	30-60	9967-74	97-40	·0097720	29-54
7	10000-00	32-68	·0032680	29-60	9870-34	32-47	·0032895	28-82
8	9967-32	32-68	·0032787	28-70	9837-87	64-94	·0066007	27-92
9	9934-64	32-68	·0032895	27-79	9772-93	32-47	·0033223	27-10
10	9901-96	65-79	·0066445	26-88	9740-46	97-73	·0100334	26-19
11	9836-17	66-01	·0067114	26-06	9642-73	195-46	·0202703	25-45
12	9770-15	99-02	·0101351	25-23	9447-27	32-69	·0034602	24-96
13	9671-13	198-04	·0204778	24-48	9414-58	163-45	·0173611	24-05
14	9473-09	33-12	·0034965	23-98	9251-14	228-83	·0247350	23-46
15	9439-96	231-86	·0245614	23-07	9022-31	130-76	·0144928	23-05
16	9208-11	132-97	·0144404	22-63	8891-55	296-38	·0333333	22-38
17	9075-14	134-45	·0148148	21-96	8595-17	134-83	·0156863	22-13
18	8940-69	470-56	·0526316	21-28	8460-34	337-07	·0398406	21-48
19	8470-13	168-06	·0198413	21-43	8123-27	372-32	·0458333	21-35
20	8302-07	269-99	·0325203	20-86	7750-96	341-45	·0440529	21-35
21	8032-08	340-34	·0423729	20-54	7409-51	375-60	·0506913	21-31
22	7691-74	512-78	·0666667	20-43	7033-91	452-68	·0643564	21-42
23	7178-96	583-93	·0813397	20-85	6581-23	313-39	·0476190	21-86
24	6595-02	448-88	·0680628	21-66	6267-84	324-20	·0517241	21-93
25	6146-15	483-41	·0786517	22-20	5943-64	396-24	·0666667	22-10
26	5662-74	419-46	·0740741	23-05	5547-40	576-35	·1038961	22-64
27	5243-28	492-66	·0939597	23-86	4971-05	184-11	·0370370	24-21
28	4750-62	387-09	·0814815	25-28	4786-93	408-19	·0852713	24-12
29	4363-54	422-28	·0967742	26-48	4378-74	336-83	·0769231	25-32
30	3941-26	387-09	·0982143	28-26	4041-92	604-40	·1495327	26-39
35	2216-96	178-79	·0806452	43-39	2418-64	—	—	37-80
40	1447-83	74-25	·0512821	60-13	1758-38	209-33	·1190476	46-00
45	1186-80	38-28	·0322581	67-93	1214-12	83-73	·0689655	60-75
50	993-91	79-51	·0800000	75-66	745-46	—	—	86-24
55	755-37	—	—	93-97	707-07	—	—	91-52
60	675-56	—	—	99-79	618-69	—	—	99-38
70	636-10	—	—	95-56	574-50	—	—	96-68
80	477-08	—	—	115-00	486-11	—	—	103-44
90	397-56	—	—	126-80	486-11	—	—	93-44
100	397-56	—	—	116-80	397-73	—	—	102-88
110	318-05	—	—	135-00	348-01	—	—	106-64
120	318-05	—	—	125-00	348-01	—	—	96-64
130	318-05	—	—	—	348-01	—	—	—
140	318-05	—	—	—	298-30	—	—	—
150	318-05	—	—	—	298-30	—	—	—
160	278-29	—	—	—	298-30	49-72	·0166667	—
170	238-54	—	—	—	198-86	—	—	—
180	238-54	—	—	—	198-86	—	—	—
190	238-54	—	—	—	198-86	—	—	—
200	198-78	39-76	·2000000	—	149-15	—	—	—
210	119-27	—	—	—	149-15	—	—	—
220	119-27	—	—	—	149-15	—	—	—
230	119-27	—	—	—	—	—	—	—
300	119-27	—	—	—	—	—	—	—

Table VI A.

## SPECIFIC DEATHS.

Comparable with *J* mice.

Cage age in days	<i>H</i> mice				<i>C</i> mice				Added daily			
	<i>l<sub>x</sub></i>	<i>d<sub>x</sub></i>	<i>q<sub>x</sub></i>	<i>e<sub>x</sub></i>	<i>l<sub>x</sub></i>	<i>d<sub>x</sub></i>	<i>q<sub>x</sub></i>	<i>e<sub>x</sub></i>	<i>l<sub>x</sub></i>	<i>d<sub>x</sub></i>	<i>q<sub>x</sub></i>	<i>e<sub>x</sub></i>
0	10000-00	—	—	28-41	10000-00	—	—	34-29	10000-00	—	—	38-29
1	10000-00	—	—	27-41	10000-00	—	—	33-29	10000-00	—	—	37-29
2	10000-00	—	—	26-41	10000-00	—	—	32-29	10000-00	—	—	36-29
3	10000-00	—	—	25-41	10000-00	—	—	31-29	10000-00	—	—	35-29
4	10000-00	—	—	24-41	10000-00	—	—	30-29	10000-00	—	—	34-29
5	10000-00	—	—	23-41	10000-00	—	—	29-29	10000-00	47-62	-0047619	83-29
6	10000-00	—	—	22-41	10000-00	—	—	28-29	9952-38	—	—	32-45
7	10000-00	—	—	21-41	10000-00	—	—	27-29	9952-38	—	—	31-45
8	10000-00	50-00	-0050000	20-41	10000-00	50-25	-0050251	26-29	9952-38	47-62	-0047847	80-45
9	9950-00	50-00	-0050251	19-51	9949-75	—	—	25-42	9904-76	95-24	-0096154	29-60
10	9900-00	50-00	-0050505	18-61	9849-75	50-51	-0050761	24-42	9800-52	95-70	-0097561	28-88
11	9850-00	300-00	-0304809	17-70	9899-24	—	—	23-54	9713-82	95-70	-0098522	28-16
12	9550-00	400-00	-0418848	17-24	9899-24	—	—	22-54	9618-12	47-85	-0049751	27-43
13	9150-00	200-00	-0218579	16-97	9899-24	51-29	-0051813	21-54	9570-27	191-41	-0200000	26-57
14	8950-00	150-00	-0167598	16-34	9847-95	102-58	-0104187	20-65	9378-86	191-41	-0204062	26-10
15	8800-00	350-00	-0397727	15-61	9745-37	153-87	-0157895	19-86	9187-46	287-11	-0312500	25-63
16	8450-00	200-00	-0236686	15-24	9591-49	51-29	-0053476	19-17	8900-35	95-70	-0107527	25-44
17	8250-00	150-00	-0181818	14-60	9540-20	102-58	-0107527	18-27	8804-65	287-11	-0326087	24-71
18	8100-00	253-13	-0312500	13-66	9437-62	102-58	-0108696	17-46	8517-54	239-26	-0280699	24-63
19	7846-88	658-13	-0638710	13-29	9335-04	259-31	-0277778	16-65	8278-28	433-17	-0523256	24-23
20	7188-75	682-79	-0921986	13-46	9075-73	419-69	-0462428	16-11	7845-11	240-65	-0306748	24-54
21	6525-96	308-31	-0472441	13-77	8656-04	419-69	-0484848	15-87	7604-47	529-43	-0696203	24-30
22	6217-64	313-49	-0504202	13-43	8236-36	160-45	-0194805	15-65	7075-04	481-30	-0680272	25-06
23	5904-15	474-44	-0803571	13-12	8075-91	267-41	-0331126	14-95	6593-75	288-78	-0437956	25-87
24	5429-71	159-70	-0294118	13-22	7806-49	374-38	-0479452	14-45	6304-97	673-81	-1068702	26-03
25	5270-01	532-32	-1010101	12-61	7434-11	374-38	-0503597	14-15	5631-16	577-55	-1025641	28-09
26	4737-69	807-56	-1704545	12-97	7059-73	646-69	-0916031	13-87	5053-60	770-07	-1523810	30-24
27	3930-13	664-25	-1690141	14-53	6413-04	986-62	-1538462	14-22	4283-53	389-41	-0909091	34-59
28	3265-88	332-12	-1016949	16-38	5426-42	493-31	-0909091	15-72	3894-12	591-51	-1518987	37-00
29	2933-76	166-06	-0566038	17-18	4933-11	274-06	-0555556	16-24	3302-61	406-47	-1230769	42-53
30	2767-70	332-12	-1200000	17-18	4659-05	328-87	-0706882	16-17	2896-13	206-67	-0714286	47-43
35	1549-91	276-77	-1785714	23-65	2726-09	174-01	-0638298	20-59	1739-19	112-21	-0645161	72-36
40	983-79	115-74	-1176471	31-38	1450-06	116-00	-0800000	31-46	1456-67	112-21	-0769231	80-99
45	636-57	57-87	-0909091	42-76	812-03	58-00	-0714286	49-71	1178-16	—	—	94-77
50	405-09	—	—	61-06	638-02	58-00	-0909091	57-59	953-75	—	—	111-31
55	270-06	—	—	84-83	522-02	—	—	64-94	897-65	—	—	113-06
60	270-06	—	—	79-83	464-01	—	—	67-87	841-54	—	—	115-42
70	270-06	—	—	69-83	406-01	58-00	-1428571	67-21	841-54	—	—	105-42
80	270-06	—	—	59-83	290-01	—	—	82-50	841-54	—	—	95-42
90	180-04	—	—	77-00	290-01	—	—	72-50	729-34	56-10	-0769231	99-53
100	180-04	—	—	67-00	290-01	—	—	62-50	617-13	—	—	107-29
110	180-04	—	—	57-00	290-01	—	—	52-50	561-03	—	—	107-37
120	180-04	—	—	47-00	232-01	—	—	54-50	561-03	—	—	97-37
130	180-04	90-02	-5000000	—	232-01	—	—	—	498-69	—	—	—
140	—	—	—	—	154-67	—	—	—	498-69	—	—	—
150	—	—	—	—	—	—	—	—	427-45	—	—	—
160	—	—	—	—	—	—	—	—	427-45	—	—	—
170	—	—	—	—	—	—	—	—	356-21	89-05	-2500000	—
180	—	—	—	—	—	—	—	—	267-16	—	—	—
190	—	—	—	—	—	—	—	—	267-16	—	—	—
200	—	—	—	—	—	—	—	—	267-16	—	—	—
210	—	—	—	—	—	—	—	—	267-16	—	—	—
220	—	—	—	—	—	—	—	—	267-16	—	—	—
230	—	—	—	—	—	—	—	—	267-16	—	—	—
240	—	—	—	—	—	—	—	—	267-16	—	—	—
250	—	—	—	—	—	—	—	—	267-16	—	—	—
260	—	—	—	—	—	—	—	—	267-16	—	—	—
270	—	—	—	—	—	—	—	—	267-16	—	—	—
280	—	—	—	—	—	—	—	—	267-16	—	—	—

Table VI B.

SPECIFIC DEATHS ONLY.

To compare with K.

Cage age in days	H mice				C mice				Added daily			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000-00	23-81	-0023810	27-05	10000-00	—	—	35-01	10000-00	22-68	-0022676	30-61
1	9976-19	—	—	26-12	10000-00	—	—	34-01	9977-32	—	—	29-68
2	9976-19	—	—	25-12	10000-00	—	—	33-01	9977-32	—	—	28-68
3	9976-19	—	—	24-12	10000-00	23-87	-0023866	32-01	9977-32	—	—	27-68
4	9976-19	23-81	-0023866	23-12	9976-13	—	—	31-09	9977-32	—	—	26-68
5	9952-38	—	—	22-17	9976-13	—	—	30-09	9977-32	22-78	-0022831	25-68
6	9952-38	47-62	-0047847	21-17	9976-13	—	—	29-09	9954-54	22-78	-0022883	24-74
7	9904-76	47-73	-0048193	20-27	9976-13	23-87	-0023923	28-09	9931-77	—	—	23-79
8	9857-03	119-62	-0121359	19-36	9952-27	—	—	27-15	9931-77	113-90	-0114679	22-79
9	9737-40	95-70	-0098280	18-60	9952-27	23-98	-0024096	26-15	9817-87	22-78	-0023202	22-05
10	9641-70	95-70	-0099256	17-78	9928-29	121-08	-0121951	25-22	9795-09	205-01	-0209302	21-10
11	9546-00	143-55	-0150376	16-95	9807-21	121-08	-0123457	24-52	9590-08	45-78	-0047733	20-54
12	9402-46	167-90	-0178571	16-20	9686-14	72-65	-0075000	23-82	9544-30	229-43	-0240385	19-64
13	9234-56	96-44	-0104439	15-49	9613-49	121-38	-0126263	23-00	9314-87	183-54	-0197044	19-11
14	9138-11	73-10	-0080000	14-64	9492-10	48-93	-0051546	22-29	9131-33	277-41	-0303798	18-48
15	9065-01	219-91	-0242588	13-76	9443-18	147-17	-0155844	21-40	8853-92	162-24	-0183246	18-05
16	8845-10	220-51	-0249307	13-09	9298-01	197-79	-0212706	20-73	8691-67	234-91	-0270270	17-38
17	8624-58	196-57	-0227920	12-41	9098-22	297-49	-0326976	20-17	8456-76	187-93	-0222222	16-84
18	8428-01	420-17	-0498534	11-69	8800-73	272-70	-0306859	19-83	8268-84	305-38	-0309318	16-22
19	8007-85	571-99	-0714286	11-27	8528-03	349-10	-0406357	19-45	7963-45	237-01	-0297619	15-82
20	7435-86	300-44	-0404040	11-10	8178-93	250-12	-0306810	19-26	7726-45	380-38	-0492308	15-29
21	7135-42	325-48	-0456140	10-55	7928-81	378-77	-0477707	18-85	7346-07	286-21	-0389610	15-05
22	6809-95	556-95	-0817844	10-03	7550-05	380-04	-0503356	18-77	7059-86	453-17	-0641892	14-64
23	6253-00	533-79	-0853659	9-88	7170-01	329-36	-0459364	18-74	6606-69	596-27	-0902527	14-61
24	5719-21	357-45	-0625000	9-75	6840-65	205-73	-0300752	18-62	6010-42	552-96	-0920000	15-01
25	5361-75	587-24	-1095238	9-37	6634-91	673-86	-1015625	18-18	5457-46	533-62	-0977778	15-48
26	4774-52	485-11	-1016043	9-46	5961-06	470-61	-0789474	19-18	4923-84	511-88	-1089604	16-11
27	4289-40	526-31	-1226994	9-48	5490-45	653-62	-1190476	19-78	4411-96	560-64	-1270718	16-92
28	3763-10	526-31	-1398601	9-73	4836-82	420-59	-0869565	21-39	3851-32	275-09	-0714286	18-31
29	3236-79	294-25	-0909091	10-23	4416-23	238-00	-0538922	22-38	3576-23	411-65	-1151079	18-68
30	2942-54	214-00	-0727273	10-21	4178-23	425-81	-1019108	22-62	3164-57	340-00	-1074380	20-04
35	1434-96	253-23	-1764706	13-14	2493-90	224-17	-0898876	31-29	1734-39	139-87	-0806452	29-78
40	866-31	28-88	-0333333	15-47	1725-20	151-33	-0877193	39-21	1105-52	58-19	-0526316	40-44
45	548-66	115-61	-2105263	18-08	1104-65	31-56	-0285714	55-04	718-17	29-92	-0416667	55-99
50	259-89	—	—	30-94	820-60	—	—	68-23	566-98	31-50	-0555556	65-35
55	231-02	—	—	29-37	725-91	—	—	71-76	440-98	—	—	78-45
60	202-14	—	—	28-07	692-92	—	—	70-50	440-98	31-50	-0714286	73-45
70	115-51	—	—	34-25	560-93	—	—	75-48	377-98	—	—	75-36
80	—	—	—	—	490-82	—	—	75-48	377-98	—	—	65-36
90	—	—	—	—	455-76	—	—	70-86	377-98	—	—	55-36
100	—	—	—	—	385-64	—	—	72-47	314-99	—	—	55-43
110	—	—	—	—	315-52	—	—	77-67	251-99	—	—	58-55
120	—	—	—	—	315-52	35-06	-1111111	67-57	220-49	—	—	56-55
130	—	—	—	—	280-47	—	—	—	188-99	—	—	—
140	—	—	—	—	245-41	—	—	—	188-99	—	—	—
150	—	—	—	—	245-41	—	—	—	157-49	—	—	—
160	—	—	—	—	245-41	—	—	—	126-00	—	—	—
170	—	—	—	—	163-61	—	—	—	—	—	—	—
180	—	—	—	—	122-71	—	—	—	—	—	—	—
190	—	—	—	—	122-71	—	—	—	—	—	—	—

Table VI c.

## SPECIFIC DEATHS.

Comparable with A to G mice.

Cage age in days	H mice				C mice				Added daily			
	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$	$l_x$	$d_x$	$q_x$	$e_x$
0	10000-00	—	—	29-06	10000-00	32-79	·0032787	46-29	10000-00	15-36	·0015361	30-08
1	10000-00	—	—	28-06	9967-21	—	—	45-45	9964-64	15-38	·0015408	29-13
2	10000-00	64-73	·0064725	27-06	9967-21	—	—	44-45	9969-25	30-86	·0030960	28-17
3	9935-28	—	—	26-23	9967-21	—	—	43-45	9936-39	30-86	·0031056	27-26
4	9935-28	65-36	·0065789	25-23	9967-21	—	—	42-45	9907-53	30-91	·0031201	26-34
5	9869-91	32-79	·0063223	24-39	9967-21	—	—	41-45	9876-61	—	—	25-42
6	9837-12	—	—	23-47	9967-21	—	—	40-45	9876-61	30-96	·0031348	24-42
7	9837-12	65-58	·0066687	22-47	9967-21	33-22	·0033333	39-45	9845-65	77-65	·0078864	23-50
8	9771-54	131-16	·0134228	21-62	9933-99	33-22	·0033445	38-58	9768-00	139-99	·0143312	22-68
9	9640-38	98-37	·0102041	20-91	9900-77	66-67	·0067340	37-70	9628-02	62-22	·0064620	22-00
10	9542-01	65-81	·0068966	20-12	9834-09	—	—	36-96	9565-80	62-32	·0065147	21-14
11	9476-20	32-90	·0084722	19-25	9834-09	66-90	·0068027	35-96	9503-48	140-22	·0147541	20-28
12	9443-30	230-32	·0243902	18-32	9767-19	67-13	·0068729	35-20	9363-27	155-79	·0166389	19-58
13	9212-97	230-32	·0250000	17-77	9700-07	100-69	·0103806	34-44	9207-47	93-79	·0101868	18-90
14	8982-65	132-10	·0147059	17-21	9599-37	33-80	·0035211	33-79	9113-68	297-53	·0226460	18-09
15	8850-55	364-63	·0411985	16-46	9565-57	101-76	·0106383	32-91	8816-15	283-38	·0321429	17-68
16	8485-92	300-68	·0354331	16-14	9463-81	68-33	·0072202	32-26	8532-78	299-67	·0351202	17-25
17	8185-24	101-47	·0123967	15-72	9395-48	172-08	·0183150	31-49	8233-10	348-99	·0423692	16-86
18	8083-77	341-09	·0421941	14-91	9223-40	34-42	·0037313	31-07	7884-11	270-77	·0343434	16-59
19	7742-68	380-22	·0491071	14-54	9188-99	240-91	·0262172	30-18	7613-34	321-24	·0421941	16-16
20	7362-46	593-18	·0805687	14-27	8948-08	174-09	·0194553	29-98	7292-10	472-03	·0647321	15-85
21	6769-28	209-36	·0309278	14-48	8773-99	348-17	·0396825	29-57	6820-07	539-72	·0791367	15-91
22	6559-92	383-82	·0585106	13-92	8425-62	209-77	·0248963	29-77	6280-35	450-14	·0731070	16-23
23	6176-09	628-08	·1016949	13-76	8216-04	141-05	·0171674	29-52	5821-22	460-44	·0790961	16-48
24	5548-02	421-37	·0759494	14-26	8075-00	250-11	·0309735	29-02	5360-78	584-51	·1090343	16-85
25	5126-65	421-37	·0821918	14-39	7824-89	358-94	·0458716	28-94	4776-27	405-06	·0848057	17-85
26	4705-28	748-57	·1590909	14-63	7465-95	582-71	·0780486	29-30	4391-22	358-58	·0820313	18-46
27	3956-71	285-17	·0720721	16-30	6883-24	404-90	·0588235	30-74	4012-64	363-21	·0905172	19-06
28	3671-54	431-95	·1176471	16-53	6478-34	640-30	·0888372	31-63	3649-43	331-77	·0909091	19-91
29	3239-60	509-60	·1573034	17-67	5838-04	384-08	·0657895	34-05	3317-66	319-35	·0962567	20-85
30	2730-00	195-00	·0714286	19-87	5453-96	309-44	·0587376	35-41	2998-31	347-37	·1158537	22-01
35	1475-79	119-66	·0810611	29-39	4177-50	232-08	·0555556	40-38	1883-75	190-28	·1010101	28-73
40	957-27	79-77	·0833333	39-08	3268-30	204-27	·0625000	45-98	1215-63	196-07	·1612903	38-39
45	638-18	39-89	·0625000	52-62	2277-32	—	—	59-92	783-32	120-51	·1538462	53-60
50	478-64	79-77	·1666667	64-67	1982-12	172-36	·0669565	63-45	522-22	—	—	74-38
55	279-20	79-77	·2857143	103-93	1551-22	—	—	75-61	461-96	20-09	·0434783	78-58
60	159-55	—	—	175-75	1421-95	—	—	77-25	378-75	—	—	90-29
70	159-55	—	—	165-75	1151-47	44-48	·0384615	85-76	241-71	—	—	128-28
80	159-55	—	—	155-75	972-99	—	—	88-65	241-71	—	—	118-28
90	119-66	—	—	195-50	972-99	48-65	·0500000	78-65	214-85	—	—	122-00
100	119-66	—	—	185-50	875-69	97-30	·1111111	76-83	214-85	—	—	112-00
110	119-66	—	—	175-50	729-74	—	—	82-03	188-00	—	—	116-93
120	119-66	—	—	165-50	632-44	48-65	·0769231	83-35	188-00	—	—	106-93
130	119-66	—	—	—	486-49	—	—	—	161-14	—	—	—
140	119-66	—	—	—	437-84	—	—	—	161-14	—	—	—
150	119-66	—	—	—	389-20	—	—	—	134-28	—	—	—
160	119-66	—	—	—	291-90	—	—	—	134-28	—	—	—
170	119-66	—	—	—	291-90	—	—	—	134-28	—	—	—
180	119-66	—	—	—	233-52	—	—	—	107-43	—	—	—
190	119-66	—	—	—	233-52	—	—	—	107-43	—	—	—
200	119-66	—	—	—	233-52	—	—	—	—	—	—	—
210	119-66	—	—	—	233-52	—	—	—	—	—	—	—
220	119-66	—	—	—	233-52	—	—	—	—	—	—	—
230	119-66	—	—	—	116-76	—	—	—	—	—	—	—
240	119-66	—	—	—	116-76	—	—	—	—	—	—	—
250	119-66	—	—	—	116-76	—	—	—	—	—	—	—
260	119-66	—	—	—	116-76	—	—	—	—	—	—	—
270	119-66	—	—	—	116-76	—	—	—	—	—	—	—
280	119-66	—	—	—	116-76	—	—	—	—	—	—	—
290	—	—	—	—	116-76	—	—	—	—	—	—	—
300	—	—	—	—	116-76	—	—	—	—	—	—	—
380	—	—	—	—	116-76	—	—	—	—	—	—	—

Table VI D.

## SPECIFIC DEATHS.

Expectations of life limited to 60 days.

Cage age in days	To compare with J			To compare with K			To compare with A to G		
	H	C	Added daily	H	C	Added daily	H	C	Added daily
0	26.25	31.14	28.58	26.48	30.16	27.37	26.26	35.31	26.66
1	25.28	30.19	27.66	25.57	29.23	26.47	25.27	34.57	25.74
2	24.31	29.23	26.75	24.59	28.29	25.52	24.29	33.71	24.81
3	23.34	28.28	25.83	23.61	27.36	24.56	23.46	32.85	23.93
4	22.36	27.32	24.91	22.63	26.49	23.60	22.47	31.99	23.04
5	21.39	26.36	24.00	21.70	25.55	22.64	21.63	31.13	22.14
6	20.42	25.40	23.20	20.72	24.61	21.73	20.72	30.26	21.17
7	19.44	24.44	22.28	19.83	23.67	20.81	19.73	29.38	20.27
8	18.47	23.48	21.37	18.93	22.78	19.85	18.88	28.60	19.46
9	17.59	22.64	20.56	18.18	21.84	19.11	18.15	27.80	18.75
10	16.71	21.68	19.84	17.37	20.96	18.19	17.35	27.11	17.90
11	15.81	20.82	19.11	16.55	20.26	17.61	16.48	26.23	17.04
12	15.32	20.86	18.38	15.80	19.56	16.73	15.55	25.51	16.32
13	15.00	18.89	17.56	15.10	18.77	16.17	14.95	24.80	15.61
14	14.35	18.02	17.00	14.26	18.06	15.53	14.34	24.16	14.79
15	13.62	17.24	16.43	13.39	17.20	15.05	13.56	23.36	14.30
16	13.20	16.55	16.04	12.72	16.52	14.36	13.14	22.72	13.79
17	12.54	15.67	15.30	12.04	15.93	13.78	12.63	21.99	13.30
18	11.80	14.86	14.90	11.32	15.50	13.14	11.80	21.50	12.90
19	11.20	14.06	14.43	10.89	15.05	12.67	11.31	20.68	12.37
20	11.21	13.47	14.30	10.70	14.73	12.09	10.89	20.34	11.93
21	11.34	13.14	13.85	10.15	14.24	11.74	10.83	19.85	11.75
22	10.92	12.82	13.97	9.62	13.99	11.25	10.18	19.76	11.75
23	10.52	12.10	14.07	9.45	13.78	11.04	9.81	19.38	11.69
24	10.44	11.53	13.82	9.29	13.49	11.15	9.90	18.82	11.70
25	9.80	11.13	14.55	8.89	12.97	11.30	9.70	18.54	12.12
26	9.89	10.73	15.29	8.93	13.46	11.55	9.55	18.53	12.25
27	10.87	10.81	17.13	8.90	13.65	11.92	10.30	19.20	12.36
28	12.02	11.74	17.98	9.09	14.52	12.68	10.09	19.52	12.61
29	12.39	11.92	20.32	9.50	14.96	12.72	10.41	20.78	12.88
30	12.17	11.66	22.36	9.42	14.89	13.43	11.30	21.38	13.27
35	15.29	13.41	32.52	11.62	19.25	18.80	13.94	23.17	15.39
40	19.12	18.96	35.60	13.12	23.01	24.65	15.89	25.39	18.59
45	25.22	29.17	41.19	14.63	31.41	33.69	18.78	32.03	24.25
50	35.73	33.73	48.15	24.22	38.40	39.33	20.80	33.25	32.20
55	50.16	38.50	49.07	22.43	40.22	47.67	30.86	39.37	33.03
60	48.50	40.62	50.51	20.86	39.28	45.17	51.62	40.18	37.21

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## BIRTH RATES AND THEIR POSSIBLE ASSOCIATION WITH THE PREVALENCE OF INFECTIOUS DISEASE.

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(With 12 Graphs.)

It will, perhaps, be interesting to students of epidemiology to compare with the delicate and complex reactions of an experimental animal population the grosser reactions of a human population. In Australia the materials have been singularly favourable for a demographical study.

A continent, as large as the United States of America, contained an aboriginal population so small as to be without significance, and so absolutely free from infectious diseases as to offer no intrusive disease factors such as in other countries have complicated colonisation. The soil and the waters were virginally unpolluted in the sanitary sense. The only diseases the colonists had they brought with them.

The climate was exceptionally favourable to health, the population for the first seventy years was for all practical purposes of pure British stock and has in fact remained, after 150 years, nearly 98 per cent. British.

Each community in turn was, at its beginning, an official or semi-official settlement small in numbers, trained to orderly public habits, with the result that vital statistics of the most complete and reliable kind are available for all parts of Australia from 1850 onwards. These statistics are so complete that no portion, however small, of the community is omitted. Written records also are available to an extent covering most of the important facts.

For the first period, from 1788 (the year of first colonisation) to 1850, the story is simple. The population at first consisted of transported convicts and military forces living on stores shipped from England. From 1788 to 1800 the typhus of the English gaols, carried into the transport ships, and incubated there by the foul conditions devised by the criminal cupidity of transport contractors, was often seen, and on some occasions most tragically seen, as the old-time ship fever. This cleared up after landing, but was replaced by dysentery and scurvy—both the result of obvious dietetic irregularities. The difficulty in establishing local crops was at first real and the settlement had to depend upon stores brought from England. These were intermittent and sometimes failed. Consequently, the settlement was on reduced rations from November, 1789, until July, 1792 (2½ years); Governor Phillip had to contribute his private stores to the public supply.

Other than these conditions, the young colony steadily grew and flourished from a community of 1035 in 1788 to a total of 44,588 in 1830.

Cunningham, Surgeon of the Royal Navy, lived two years in New South Wales and, in 1827, wrote an account of the colony in which this general description occurs:

The extraordinary healthiness of the climate of New South Wales must be of no trifling importance in the eyes of a European, considering how unhealthy most other countries are. Intermittents, remittents, typhus, scarlet fever, smallpox, measles, whooping-cough, and croup are here unknown...on reaching the age of puberty, phthisis is liable to supervene from the rapid sprouting out in stature of our youths at this period: but the European phthisis is uniformly cured, or at least relieved, by a removal hither, if early resorted to.

In 1794 ophthalmia was epidemic, in 1824 mumps appeared, in 1820 and 1826 influenza was prevalent—"fatal chiefly to infancy and old age." In 1828 "a kind of whooping-cough made its appearance in Sydney and spread over the whole colony. It proved fatal to a few children; the numbers are very small, compared to those attacked."

That is the epidemiological story of a considerable community over forty-two years.

The composition of the population showed slow changes. During the convict period there was great excess of males over females. At the commencement of the settlement in Sydney this excess was so great that Governor Phillip had instructions to procure native women from the adjacent islands for the use of the male convicts. This was not done. Very soon after the commencement, in 1797, Phillip reported that "the vast number of women for whom we have very little work are a heavy weight upon the store of Government." Up to 1850 the percentage of children in the population was much lower than in a normal community.

The second period from 1830 to 1850 was characterised by the establishment of the colonies of Victoria, Queensland, South Australia and Western Australia, and by quiet development of the population along agricultural and pastoral lines.

The convicts transported before 1810 began to die in considerable numbers, producing a heavy death rate between 1832 and 1842; but still Governor Gipps could report in 1845 that "the adult males in the Colony are still more than double the females." Between 1838 and 1842 there was a great wave of voluntary immigration, adding in those five years a total of 86,816 persons to the community of New South Wales, which before had comprised only 113,437 persons.

Influenza was very severe in 1836 and 1838: this being the first definite occasion on which Australia shared in a general epidemic manifestation of the disease in several countries.

Scarlet fever made its appearance in Tasmania in 1833 and in New South Wales and Victoria in 1841.

Measles appeared first in the different states between 1850 and 1857.

Whooping cough reappeared for a brief epidemic.

Smallpox, from an unknown source, caused a very high mortality amongst the aborigines inland, but did not attack the white population.

Typhus fever—aptly named the “gaol-fever”—had declared itself severely under the conditions of gross overcrowding and insanitation in the convict establishments of Tasmania; but, as these predisposing conditions were not present in the convict stations in Western Australia, typhus did not occur there.

Such were the conditions in 1850 in Australia. There were six settlements forming the embryonic stages of six great colonies, with populations which were scattered as to space, few as to numbers, and ill-balanced as to age-constitution, which were free for the most part from the commoner epidemics, these appearing intermittently, disappearing totally in the intervals, and which knew two types of pulmonary tuberculosis—the acute, rapidly developing adolescent type of “our youths” and the more chronic “European phthisis.”

After 1850 the whole picture was abruptly changed by the discovery of gold.

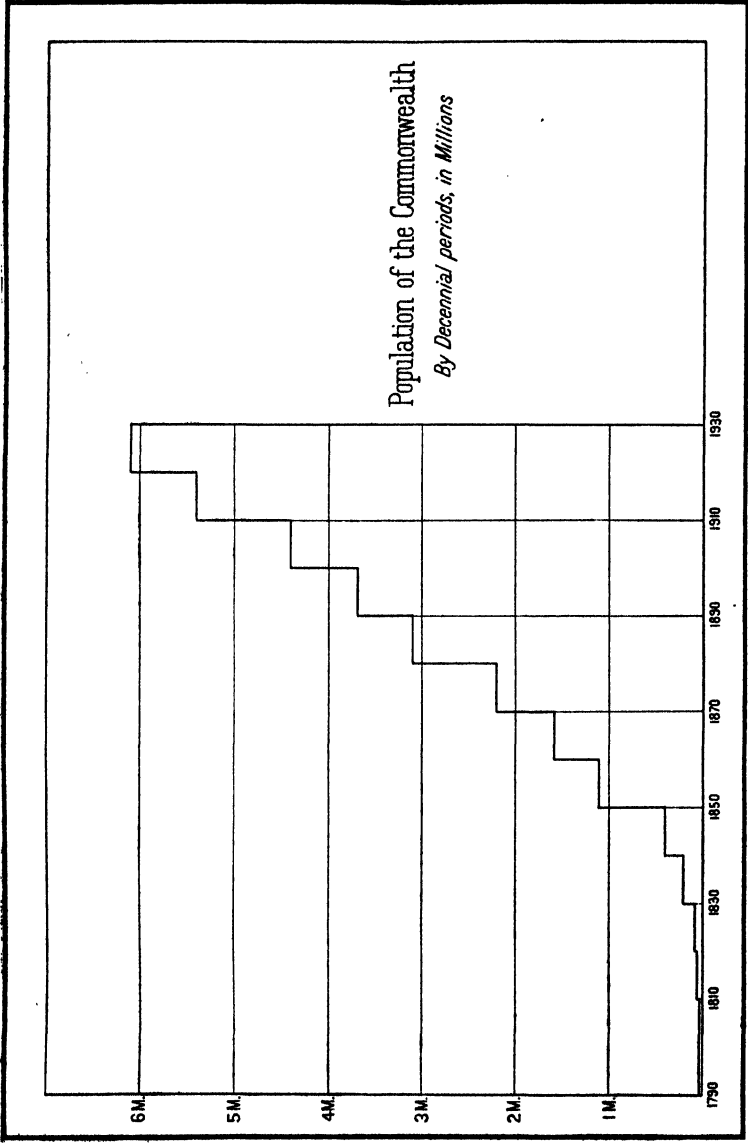
The population of all Australia in 1850 was 405,356, in 1856 it was 876,729, and by 1860 it was 1,145,585. This great human wave was composed principally of young adults who, apart from considerations of economic and social value, distorted the proportional age-constitution of the population by providing a great excess of young adults.

The winning of gold from the ground soon became unreliable as a means of livelihood and other industries were developed. The prosperity attending these became so great that, in the decade 1880–90, another great wave of migration brought to Australia a net immigration total of 382,741 persons. Again, in the five years 1909 to 1913 inclusive, organised immigration added a total of 281,193 persons. The population development of Australia as a continent is shown in Graph I. From this are seen clearly the three great accessions of the “’fifties,” the “’eighties” and between 1910 and 1920. These three waves of the “’fifties,” the “’eighties” and around 1910 were notable points in the population development of Australia.

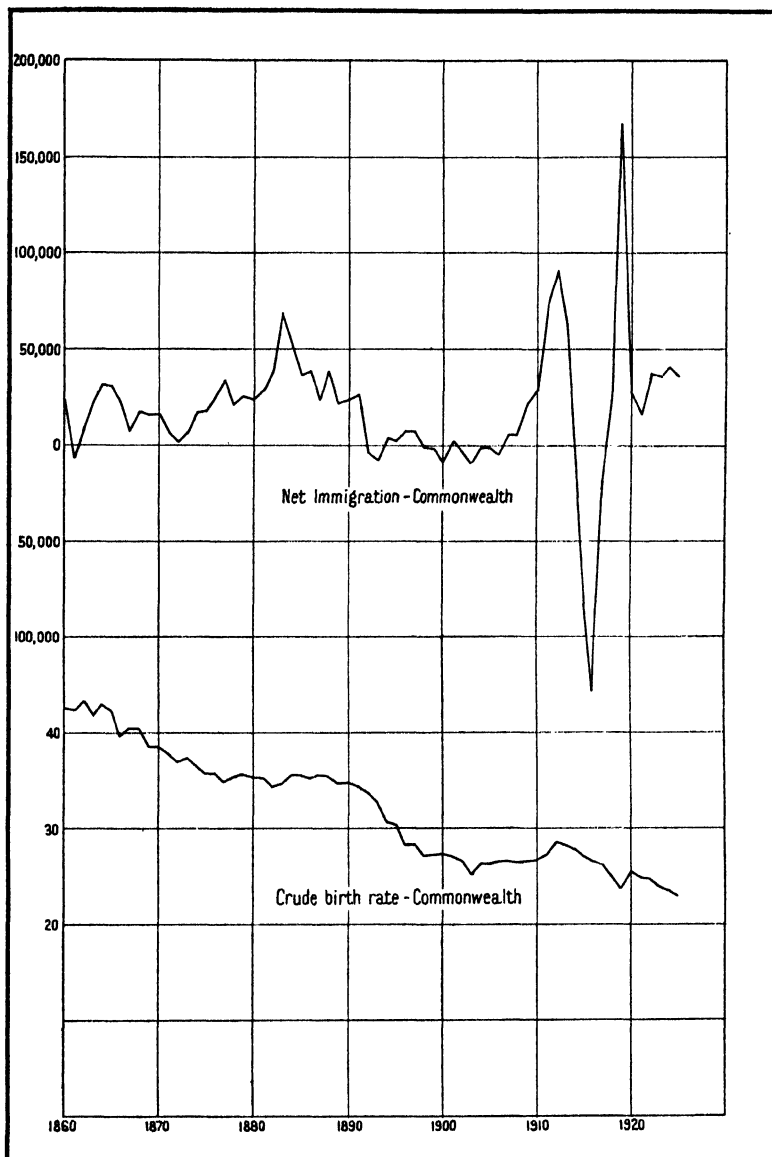
The periods 1860–1920 are shown on Graph II—as comparable statistics are not available for all the Australian States between 1850 and 1860, the graph commences only with the year 1860.

From the same graph it can be seen that these two periods of immigration increment to the population were immediately followed by periods of increased birth rate. Although not shown on the graph, this increase was seen to an even greater extent in the ‘fifties.

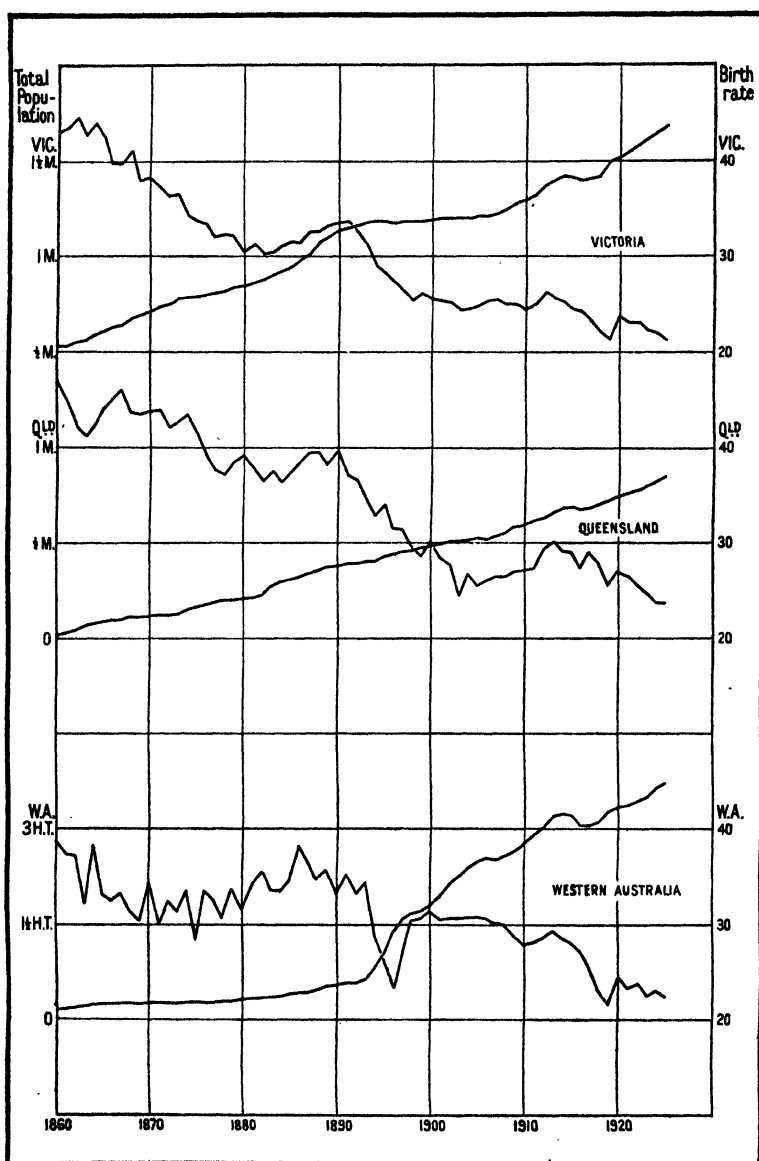
These periods illustrate the results accompanying immigration from outside Australia. Two other occasions also illustrated the same association of events following internal migration. In the early ‘eighties gold was discovered in Queensland, and in the early ‘nineties in Western Australia. Graph III shows the population and birth rate in each of these two states and illustrates the



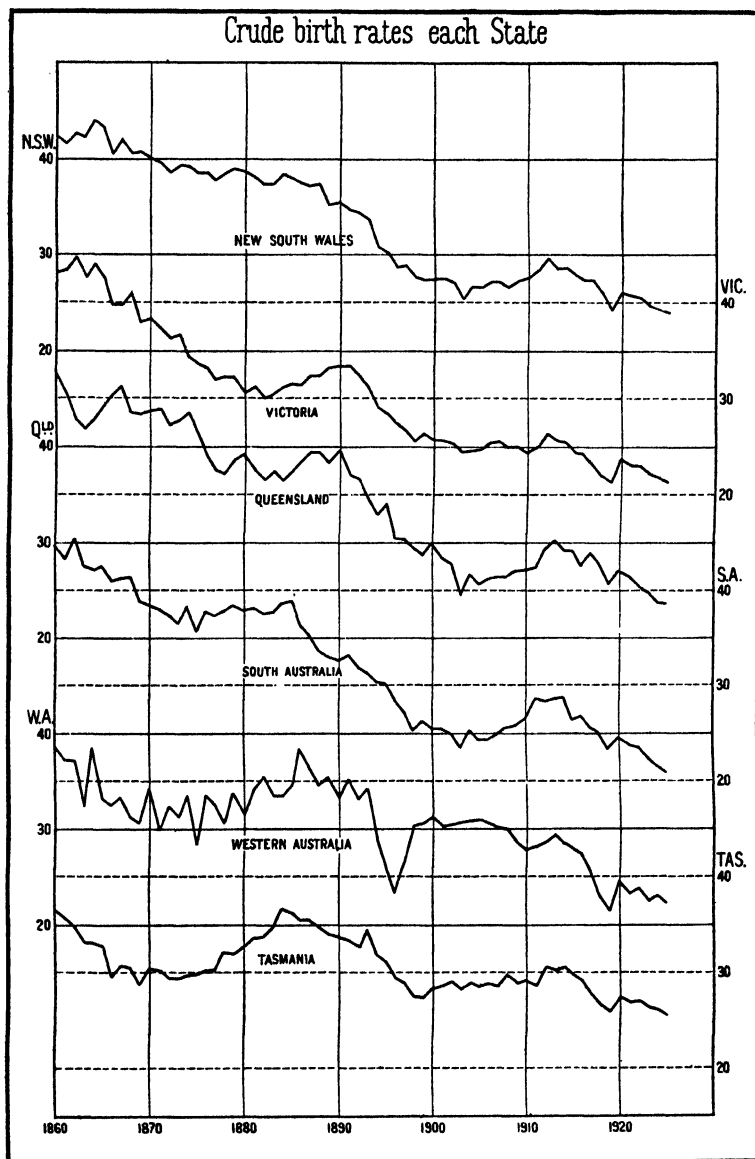
Graph I. Population development in Australia as a whole.



Graph II. Absolute net immigration and crude birth rates, for the years shown, for Australia as a whole.

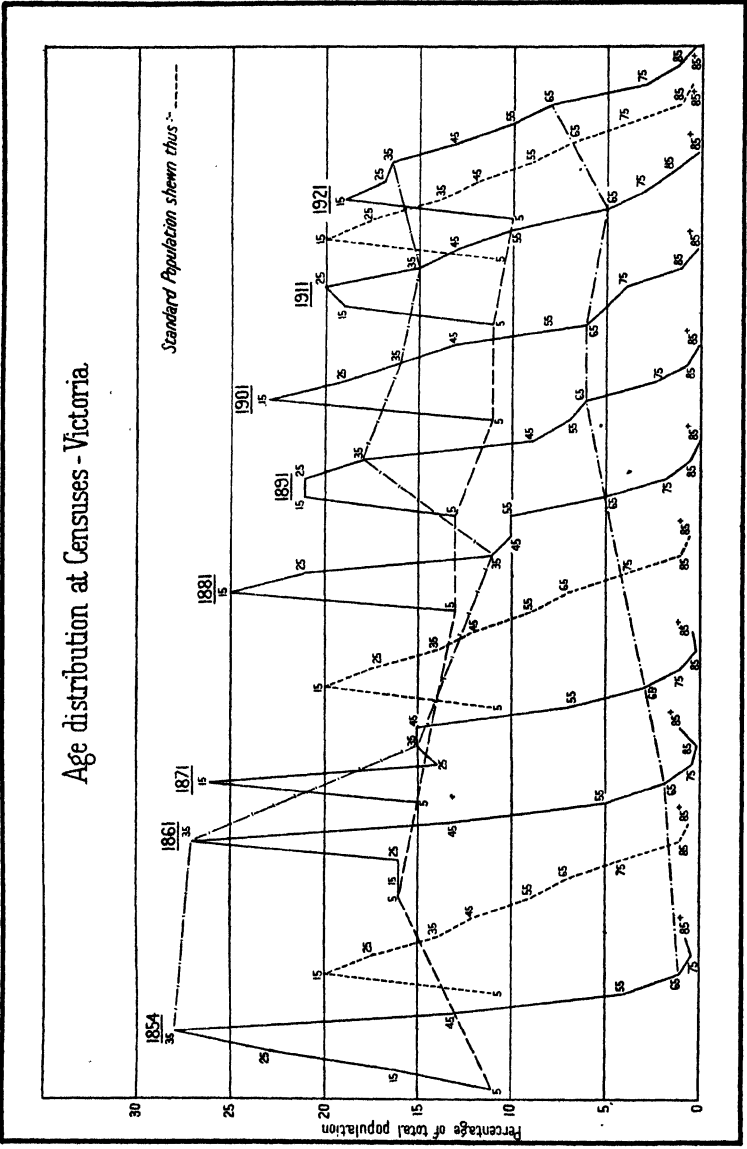


Graph III. Population and birth rates for the years shown in Victoria, Queensland and Western Australia.

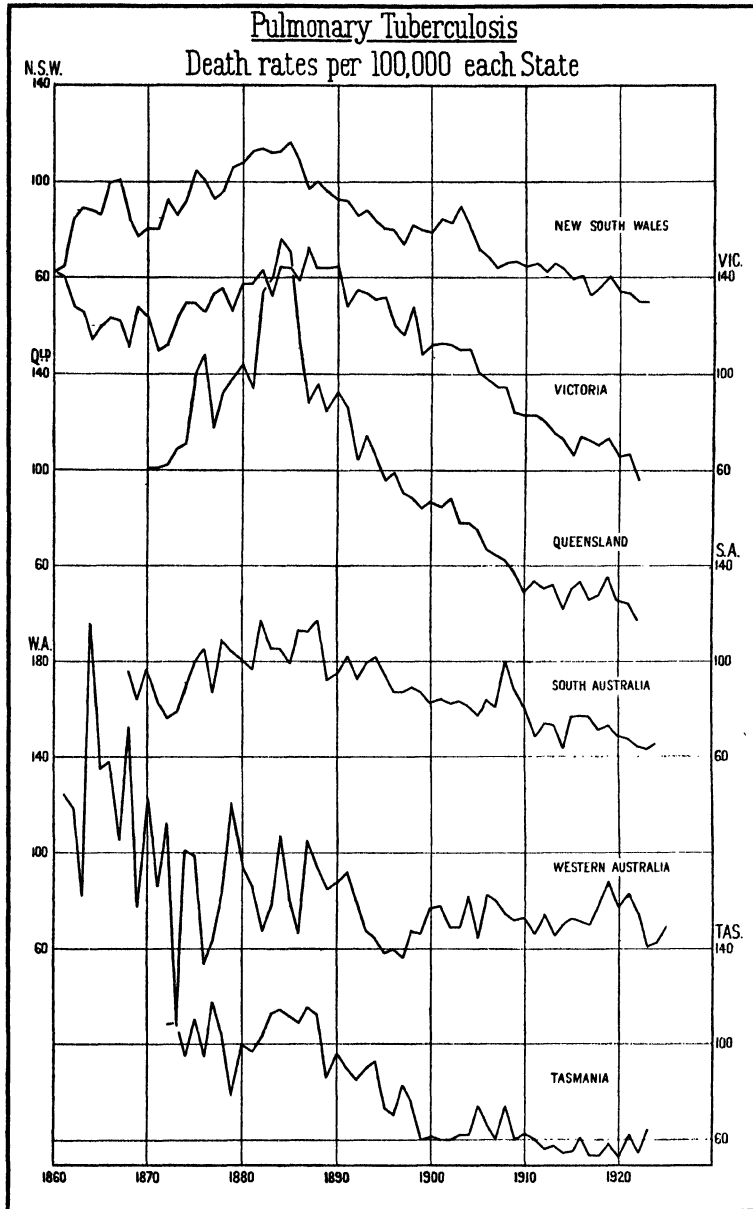


Graph IV. The course of the birth rate in each State showing the high level in the 'sixties, the rise in the 'eighties and again around 1910.

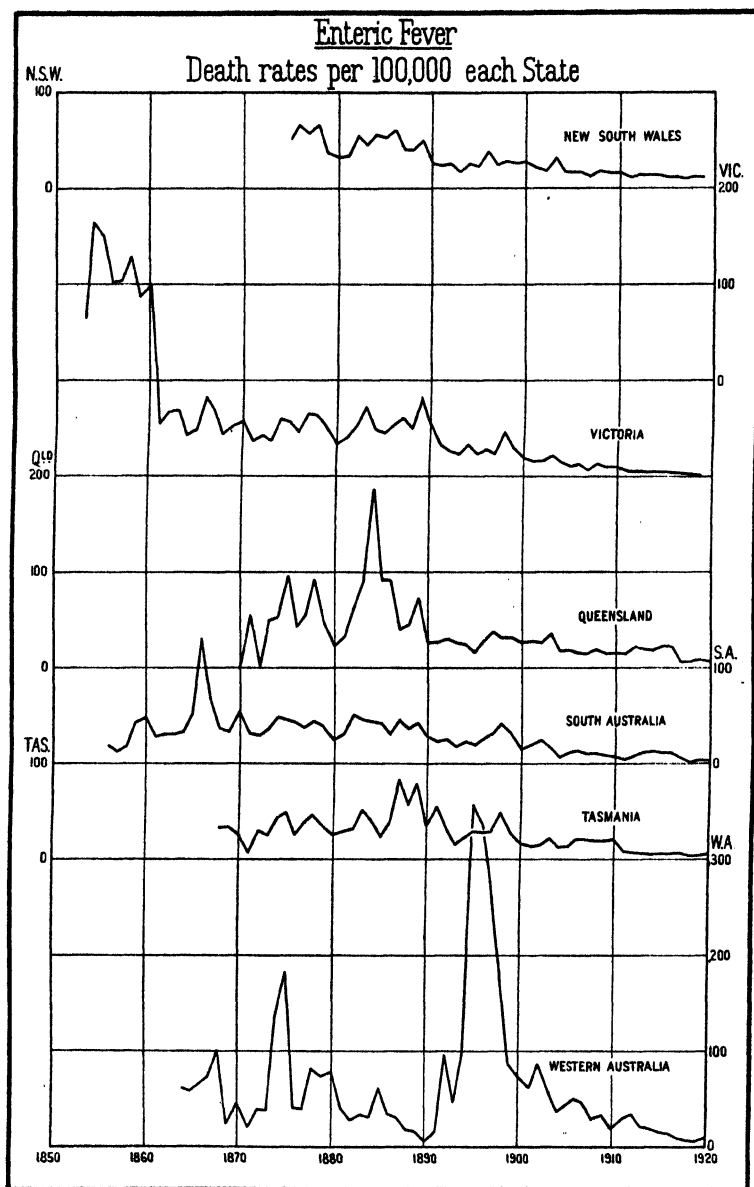




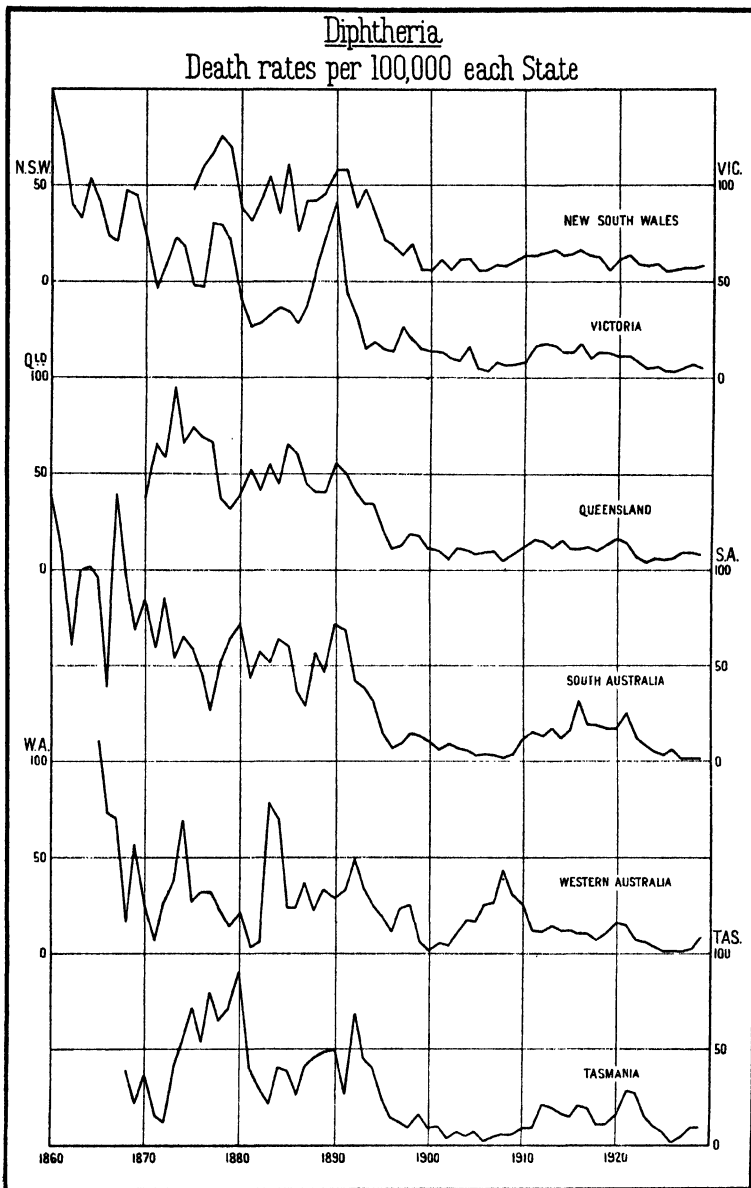
Graph V. Percentage age-composition of the population of Victoria at each Census 1854-1921. The percentage age-composition of the "standard" population is shown for comparison in three places.



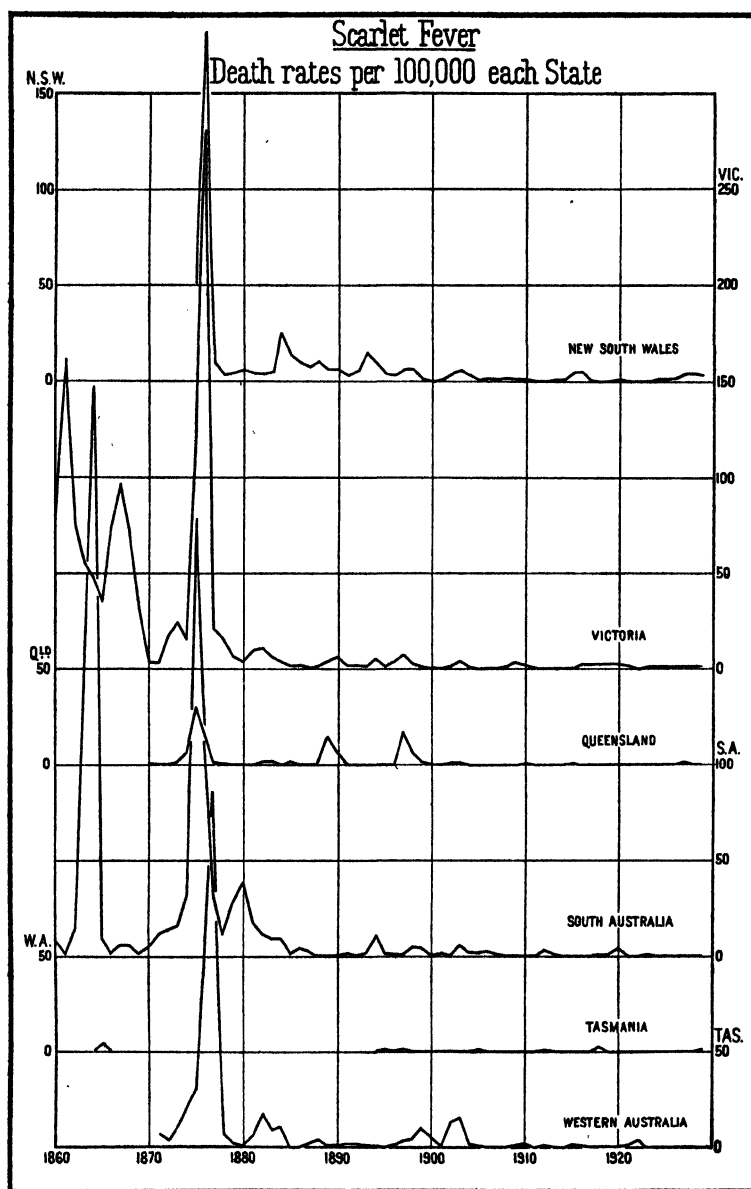
Graph VI. Death rates from pulmonary tuberculosis in each State.



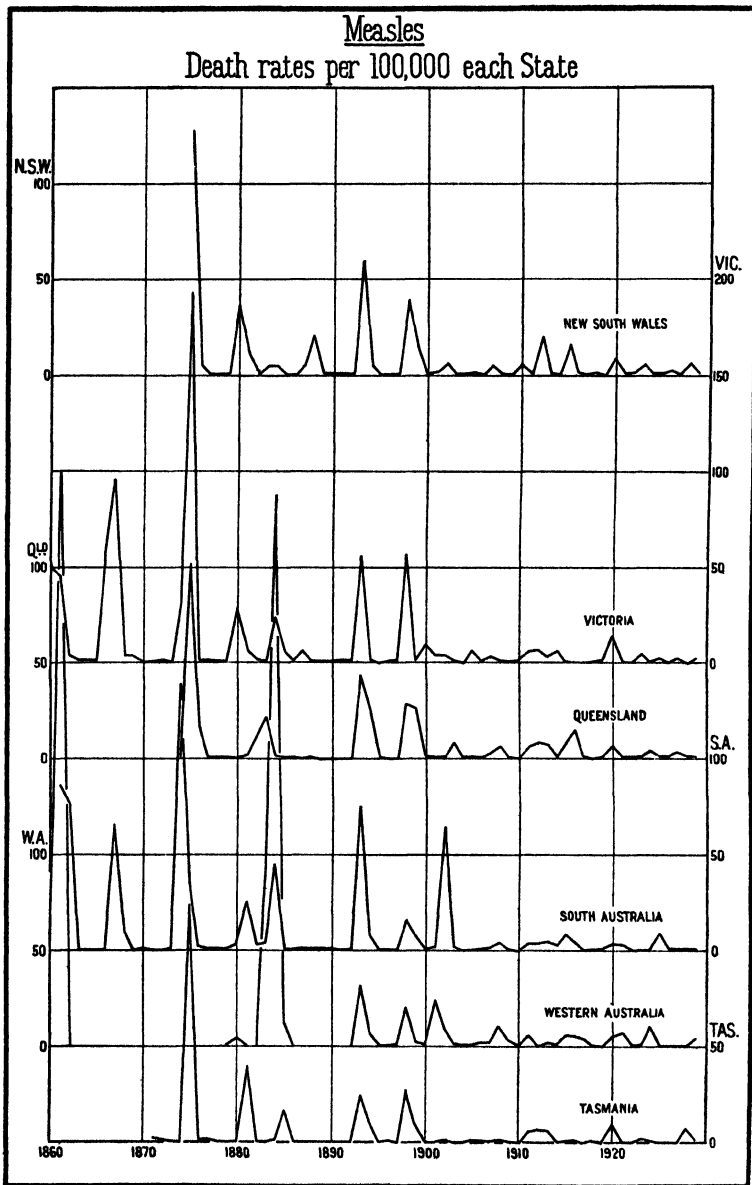
Graph VII. Death rates from enteric fever in each State.



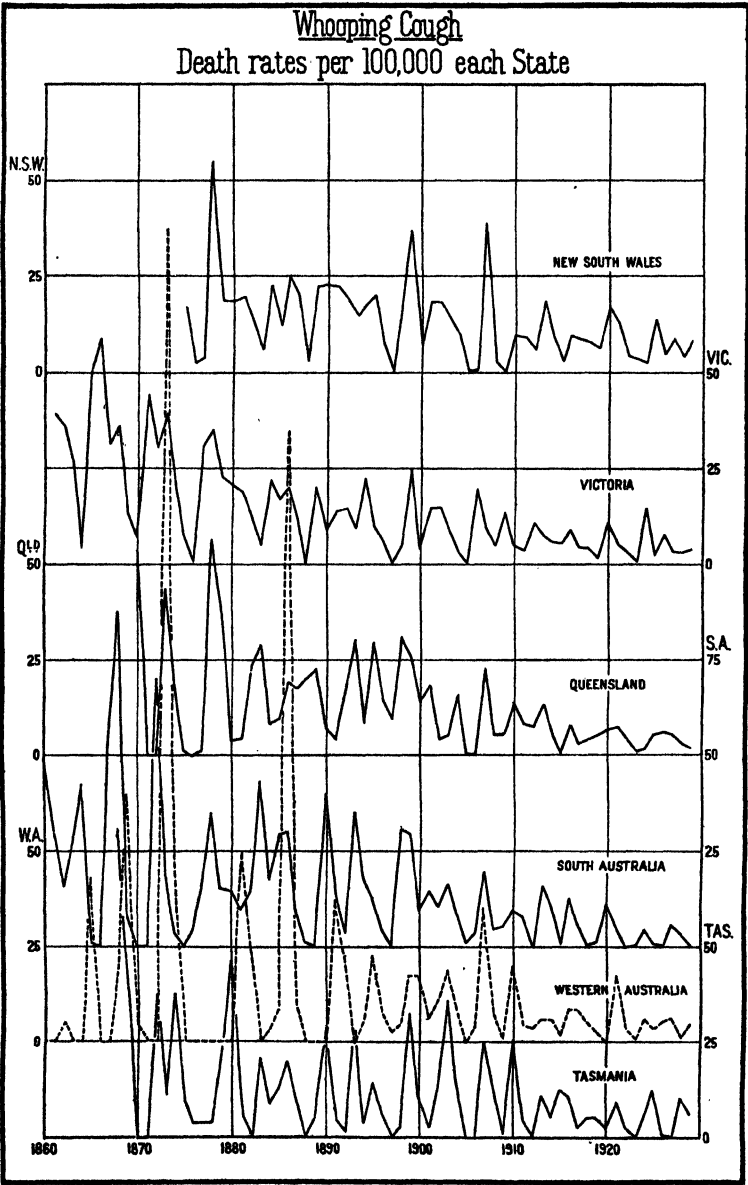
Graph VIII. Death rates from diphtheria in each State.



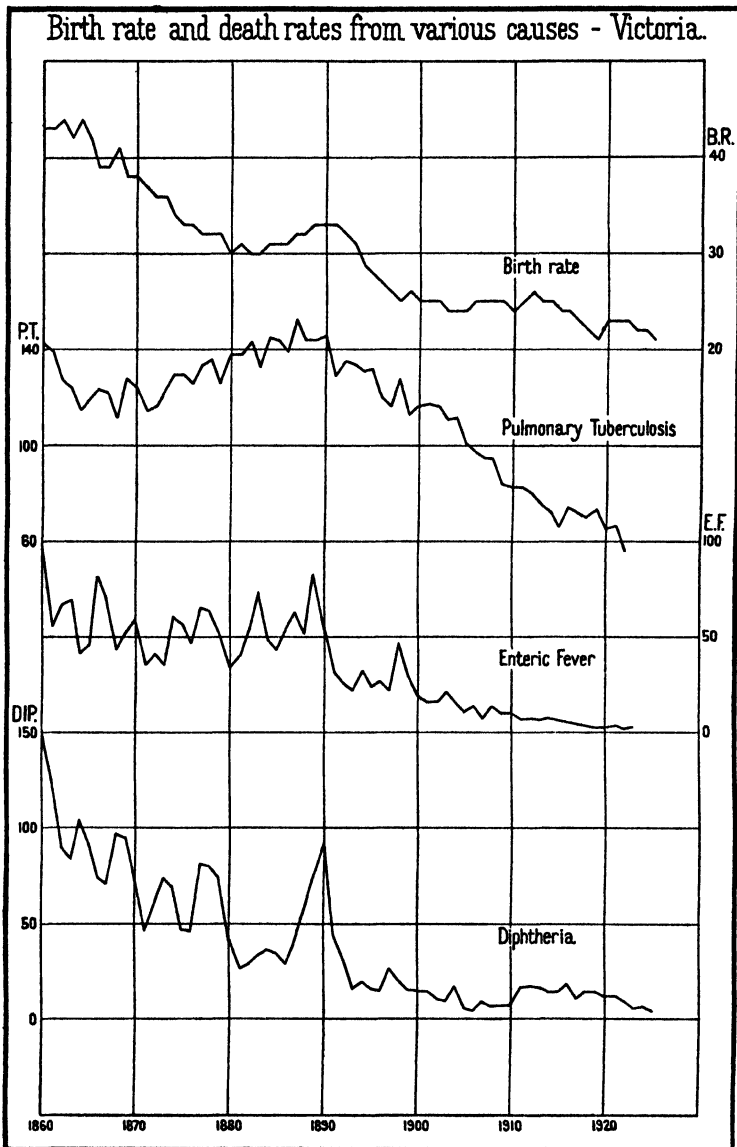
Graph IX. Death rates from scarlet fever in each State.



Graph X. Death rates from measles in each State.



Graph XI. Death rates from whooping cough in each State.



Graph XII. The course of the birth rate and of the death rates from pulmonary tuberculosis, enteric fever and diphtheria in Victoria.



rise in the birth rate following on the increase in population. In Western Australia the sudden fall in the birth rate caused by the abrupt introduction of a large numerical mass of adults is very striking. The course of the birth rate for each State is shown in Graph IV.

The effect of these changes upon the age-constitution of the population is well seen in Graph V. Here the percentage age-composition of the population of Victoria at each census is shown, the composition of the international standard population being inserted three times for comparison.

In 1854 and 1861 the 15-25 and 25-35 groups are greatly in excess of other ages and of their percentage status in the standard population: the 55-75 group was much below its normal place; and the 0-5 group had increased in relation to other age-groups and had reached a point well above its normal position in the standard population.

The immigration wave of the 'eighties saw the same changes to a less degree, while in the period 1909 to 1913 these changes are barely visible on the graph.

Simply told, this is the story of an exodus of young people to a new land where they had children and these children grew up.

Experience in Australia indicates pulmonary tuberculosis and enteric fever as the two great diseases of young adult life and the respiratory infections, diphtheria, scarlet fever, measles and whooping cough as the diseases of childhood. In this respect experience is similar to that in other countries.

On this we should expect to find enteric fever and pulmonary tuberculosis causing deaths in definite relationship with the presence in the community of persons at susceptible ages. The recorded experience prior to 1850 has been quoted.

Reference to Graph VI will show the death rates from pulmonary tuberculosis in the various states. Omitting from consideration the immigrants themselves, their infants—the 1850 to 1860 group—would be growing during the susceptible ages for both enteric fever and pulmonary tuberculosis during the years prior to 1890—the enteric fever prevalence (see Graph VII) attacking the group at younger ages than the pulmonary tuberculosis. Graphs VI and VII show the very special prevalence of enteric right up to 1890 in New South Wales and Victoria and the notable rise in pulmonary tuberculosis in New South Wales, Victoria, Queensland and South Australia. The immigrants themselves suffered from enteric fever, as is seen by reference to Graph VII: the excessive mortality of the 'fifties in Victoria, of the 'eighties in Queensland, and of the 'nineties in Western Australia being, in fact, part of the familiar history of Australia.

To be consistent this association should be seen in the minor waves as well as in the major. To some extent this was so, as evidenced by the minor enteric wave culminating in 1889 in Victoria and by the suggestive halt in the decline of the curve in tuberculosis mortality between 1915 and 1921 which followed the birth rate rise of the 'eighties after an interval comparable with that

between the birth rate rise of the 'fifties and the tuberculosis mortality rise of the 'eighties.

When the respiratory infections are considered, the course of the diphtheria rate presents some interesting features. As in England, diphtheria was for all practical purposes unknown in Australia before 1860. On its introduction it found an abundant susceptible population of the most suitable age and struck hard. Thereafter it declined until, in consonance with the rise in the birth rate in the 'eighties, it showed a further definite rise in Victoria, South Australia and Tasmania and to a less extent in Queensland. The course of the death rate is shown in Graph VIII. The rise in the death rate in consonance with the rise in the birth rate around 1910 is also evident in most of the states.

In Western Australia, however, the course of events is somewhat different, although still significant. After the gold rush of the 'nineties, large new populations in large new centres had developed. In these young adult populations many children had been born, but these centres were separated by long distances often with infrequent communication. The slow rise in the diphtheria death rate between 1900 and 1910 is consistent with these conditions.

The rise in the diphtheria death rate in all the states between 1910 and 1925, even with the general use of antitoxin, specially calls for consideration in view of the rise in the birth rate at this period.

*Scarlet fever* has followed a striking course in Australia. This course is shown in Graph IX. In Queensland and Tasmania it has never called for any serious attention. In Victoria and South Australia it was a serious disease between 1850 and 1876, but in New South Wales and Western Australia it was not an important disease until 1875-6. In those two years the states of New South Wales, Victoria, South Australia and Western Australia experienced scarlet fever in epidemic form of tragic extent and virulence. Since 1890 the disease has not been a serious cause of death in Australia, although it continues to prevail with disabling complications.

*Measles* has shown a definitely epidemic course in Australia. In Western Australia, especially, there was a great epidemic in 1860-1 after the subsiding of which no death from this disease was recorded until 1884 when the disease appeared producing a severe mortality in a population, both black and white, which seemed to be quite unprotected. Graph X shows how, in each state, the disease appeared in epidemics, dying out completely in the intervals, until 1900 when the disease became stabilised at a very low death-rate level. When this course is compared with the course of the birth rate, it is obvious that the high mortality levels prevailed while the birth rate was high and as soon as the birth rate fell below 30 the death rate was stabilised at the low level. The minor variations in accord between the two rates even at these lower levels are seen in New South Wales between 1910 and 1920, in Victoria in 1920, and in Queensland and South Australia between 1910 and 1915.

*Whooping cough* has exhibited a less characteristic course. The behaviour of the disease is seen in Graph XI. It is difficult to say more than that, while

the birth rate was high, the death rate was also high and, after 1900, when the birth rate was lower, the death rate was also generally at a lower level.

In Graph XII the course of the birth rate and the death rates from pulmonary tuberculosis, enteric fever and diphtheria in Victoria have been brought together so that the course of the events above narrated may be more clearly seen.

This brief survey has been prepared with the object of presenting the picture of a human population under closely observed conditions during eighty years. This population has been affected by fluctuations of a magnitude only possible in a young country with a small initial population. Australia now has a stable population of such a size that immigration is not likely to affect its quality to any material extent and its age-constitution is now approximating the standard.

All the evidence here presented indicates that the stage of the wide-amplitude variations in birth and death rates is, for this population, now over and it must be studied on the facts available, no new opportunity for recording and studying similar phenomena being likely.

In considering the story the other factors, such as social, dietetic and sanitary conditions in a new country, especially during a gold rush, as well as the specific attributes of the diseases mentioned, etc., have not been overlooked. They could have been discussed at great length without much profit. It has seemed well to present the facts here assembled as, perhaps, at least a suggestive contribution to the discussion on influences associated with epidemics now somewhat inconclusively proceeding.

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# THE SPERMICIDAL POWERS OF CHEMICAL CONTRACEPTIVES.

## III. PESSARIES.

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### INTRODUCTION.

My first paper (1930) in this series contained a general introduction and an account of a preliminary investigation of a few pessaries with guinea-pig sperms. The second paper (1931) was concerned not with pessaries, but with chemically pure substances, free from any vehicle. In this third paper I return to pessaries. First I describe some experiments performed with guinea-pig sperms in continuation of those reported on in my first paper. I then present an account of a technique for pessaries using human sperms, and of the results obtained with it. Finally, experiments on the foam-producing pessaries in the absence of the foam-producing substances are described. The next paper in the series will deal with more pure substances.

I wish to thank the Hon. Mrs Marjorie Farrer and the Birth Control Investigation Committee once more for continued support. The work described in this paper could not have been performed had not Dr C. P. Blacker given invaluable help in many ways. Prof. E. S. Goodrich, F.R.S., has kindly allowed the whole of this investigation to be carried out in the Department of Zoology and Comparative Anatomy at Oxford. I want to thank him for his interest in the work.

## THE PESSARIES INVESTIGATED.

Some of these were described in the first paper, but it will be convenient to describe them all here, especially as I now have more accurate information as to some of them. The makers of all the pessaries have most kindly communicated the quantitative analyses of them to me, but they do not allow me to make these analyses known to anyone else, except in the case of the quinine and chinisol pessaries.

The qualitative analyses are not secret.

*Quinine.* The pessary is shaped like a solid flattened thimble. It weighs 2.08 gm. It consists of 0.324 gm. of quinine bisulphate in cocoa-butter. It is made by Messrs Lambert.

*Double-strength quinine.* This is the same as the quinine pessary, except that it contains 0.648 gm. of quinine bisulphate. It is made by Messrs Lambert.

*Chinisol.* This is the same as the quinine pessary, except that it contains 0.195 gm. of potassium oxyquinolin sulphate (chinisol) instead of quinine. It is made by Messrs Lambert.

*Lactic acid.* The pessary is shaped like a solid flattened thimble. It weighs 1.58 gm. It consists of lactic acid in cocoa-butter. It is made by Messrs W. H. Martindale.

*Contraps.* This is a large spherical pessary weighing 10.9 gm. It consists of magnesium sulphate, lactic acid, quinine bisulphate, glycerine, tragacanth and water. There is no cocoa-butter nor foam-producing substances. It is made by Messrs Docker.

*Quinine urea hydrochloride.* This is a minute tablet weighing 0.17 gm. It contains only quinine urea hydrochloride and sodium chloride. There is no cocoa-butter nor foam-producing substances. It is made by Messrs Parke, Davis.

*Semori.* This is a foaming tablet weighing 1.04 gm. It consists of dioxyquinolin sulphate, potassium borotartrate, sodium bicarbonate and tartaric acid. It is made by Messrs Luitpold Werk in Munich.

*Speton.* This is a foaming tablet weighing 1.20 gm. It consists of sodium dichlorylsulphamidbenzoate, lactose, starch, French chalk, sodium bicarbonate and tartaric acid. It is made by Messrs Temmler in Berlin.

*Finil.* This is a thin foaming tablet. Two are directed to be used together. The weight of two is 1.30 gm. The constituents are dioxyquinolin sulphate, boric acid, burnt alum, starch, egg albumen, sodium bicarbonate and tartaric acid. Finil is made by the Pharmazeutische Fabrik in Munich.

*Monsol.* This is a foaming tablet weighing 0.97 gm. It contains powdered quillaia bark, monsol fluid, gum extract, sodium bicarbonate and potassium hydrogen tartrate. Monsol fluid contains substances allied to cresol. This tablet is made by the Mond Staffordshire Refining Co., but is not yet on the market.

## EXPERIMENTS WITH GUINEA-PIG SPERMS.

In the first paper in this series it was shown that if a quinine or cocoa-butter pessary is thrown into 7.5 c.c. of guinea-pig sperm suspension at the temperature of the body, and left there for a quarter of an hour, the activity of the sperms is affected little or not at all.

This gave rise to the supposition that the inefficiency of the pessary might be due simply to the slowness of the diffusion of the quinine or chinisol out of the cocoa-butter. Accordingly a series of experiments was performed, in which the pessary was left in glucose-saline solution for 12 hours at the temperature of the body, with occasional shaking, before the introduction of the

sperms. As before, one pessary was allowed to 7.5 c.c. of fluid. Although the pessary was of course melted, the sperms were scarcely or not at all affected in a quarter of an hour.

The conclusion reached was that the inefficiency of the pessary was due to the low spermicidal power of quinine bisulphate and chinosol. Since the first paper was written, I have disproved this conclusion, as follows.

A series of experiments was carried out in exactly the same way as in the first series, except that instead of introducing one pessary into 7.5 c.c. of sperm suspension, 0.324 grm. of finely powdered quinine bisulphate or 0.195 grm. of finely powdered chinosol was introduced instead. These are the actual amounts contained in one pessary. The results, which are most remarkable, are recorded below. The activity of the sperms, after a quarter of an hour with the quinine or chinosol, is indicated by a system of grading which is explained at length in my second paper. III indicates that the majority of the sperms were active; 0 indicates that not a single active sperm was seen in ten microscopical fields of a  $\frac{1}{8}$  in. objective with No. 2 eyepiece. I, I+, II and II+ indicate intermediate grades of activity. In the experiments recorded below, the control sperms were III or III+ in each experiment.

The results were as follows:

Quinine	0	0	0
Chinosol	0	0	0

Not a single movement in a single sperm was seen in any of the six experiments.

This experiment was performed at *S* (standard) concentration, *i.e.* in the proportion of one pessary to 7.5 c.c., which is postulated as being the usual amount of fluid in the vagina after coition. The next series of experiments was performed on *S/10* concentration. The technique was the same, except that 0.032 grm. of quinine bisulphate and 0.02 grm. of chinosol were used instead of the 0.324 grm. and 0.195 grm. respectively. Three experiments were again performed with each pessary, the control sperms being III or III+ in each case. The results were as follows:

Quinine	0	0	0
Chinosol	0	I	I+

This shows that although a pessary of quinine or chinosol has scarcely any or no effect on sperms, even when it has been melted for 12 hours, yet it contains ten times as much quinine as suffices to kill every sperm in a quarter of an hour, or ten times as much chinosol as suffices to kill the great majority of the sperms. I cannot account for this paradoxical result. It would be understandable if quinine bisulphate or chinosol were more soluble in cocoa-butter than in water; but they are quite insoluble in cocoa-butter, and are only suspended in it.

The following table shows the results of all the experiments which have been performed by the standard technique for comparing pessaries, using

guinea-pig sperms. Most of these results were recorded in my first paper, but they are all brought together in one place for convenience. The pessaries are arranged in the order of their spermicidal powers. The control sperms were III or III+ in each case. The result of the experiment was not recorded if the control sperms were less active than this.

	At <i>S</i> concentration				At <i>S</i> /10 concentration		
Quinine urea hydrochloride	0	0	0	0	I	I	I +
(Semori	0	0	0	0	I	II	II +
(Speton	0	0	0	0	I	II +	II +
Chinosol	I +	III	III	III +	Not tested		
Quinine	II +	III	III	III	Not tested		

#### EXPERIMENTS WITH HUMAN SPERMS.

##### *Brief description of the technique.*

Guinea-pig sperms are extremely convenient to work with, but it was felt to be essential to carry out a series of experiments using human sperms. This was made possible by two men who volunteered as donors. Both men are of young middle age and the fathers of families, so their sperms may be regarded as normal.

In the experiments with human sperms every effort was made to represent actual conditions as closely as possible. The whole experiment was carried out on the scale of one-fifth, in order to economise semen. Thus, instead of trying one pessary to 2.5 c.c. of artificial vaginal fluid and 5 c.c. of semen, which is postulated as being the standard (*S*) concentration, one-fifth of a pessary was used with 0.5 c.c. of artificial vaginal fluid and 1 c.c. of semen.

The following is a brief description of the technique, for the benefit of those who do not care to follow the detailed description.

0.5 c.c. of artificial vaginal fluid (a neutralised glucose solution) is placed in a glass specimen tube in a damp chamber in a thermostat maintained at the temperature of the body. 1 c.c. of human semen is placed in another tube in the same damp chamber. When the fluids have warmed up, one-fifth of a pessary is thrown into the artificial vaginal fluid. A quarter of an hour later the warm semen is transferred to the same tube as the artificial vaginal fluid and the one-fifth pessary. Half an hour later the sperms are examined under the microscope. The result of the experiment is not recorded unless the control sperms are active (II+, III or III+).

This experiment was performed four or five times with each make of pessary. Those pessaries which were shown by this experiment to be effective were tested again at *S*/10 concentration.

The details of the general technique and of the special techniques required for certain of the pessaries are given below.

*Results.*

The following is a summary of the results of the experiment. The pessaries are arranged in the order of their spermicidal powers.

	At <i>S</i> concentration				At <i>S</i> /10 concentration				
Semori	0	0	0	0	0	0	I	I	I +
Quinine urea hydrochloride	0	0	0	0	0	I	I +	I +	
Monsoi	0	0	0	0	I	I	I	I	I +
Speton	0	0	0	0	0	I +	II	II	II
Finil	0	0	0	I	I	I +	I +	II	II
Chinosol	0	0	0	I					
Double-strength quinine	II +	II +	III	III					
Quinine	II +	III	III	III	III				
Lactic acid	II +	III	III	III +					

A complete investigation of contraps was not made, as its very large size introduced difficulties. Only a minute part dissolves when one-fifth of it is placed with 0.5 c.c. of artificial vaginal fluid. In two experiments at *S* concentration it reduced the activity of the sperms to I. It would have been equally effective at *S*/10 concentration, for the same amount of it would have dissolved.

It seems worth mentioning that the mean amount of semen produced at each ejaculation by one of the donors was 6.7 c.c. This is based on the measurement of 23 ejaculates. The maximum amount was 10.5 c.c. and the minimum 4.5 c.c.

*Full description of the technique.*

The following is a detailed account of the technique, which may be applied, with the specified modifications, to all pessaries of reasonable size. In order to shorten this account, several references are made to the second paper in this series, in which a very detailed account is given of a somewhat similar technique, for comparing the spermicidal powers of pure substances.

(1) A thermostat is maintained at 37° C. It contains coverslips, pipettes and a damp chamber. The latter, which contains a rack of small specimen tubes, is described and figured in my second paper.

(2) A hot stage, regulated thermostatically to 37° C., is arranged on a microscope.

(3) The average weight of the pessary to be investigated is determined.

(4) One-fifth of a pessary is weighed out, and set aside in a corked specimen tube, sealed with wax, until semen is available.

(5) Some ice and a corked specimen tube are placed in a vacuum flask, the cork of the specimen tube having been impregnated with paraffin wax.

(6) Semen is caught in a rubber sheath at coition.

(7) The contents of the sheath are shortly afterwards transferred to the corked specimen tube, and left within the vacuum flask until required for the experiment.

(8) A neutral fluid, isotonic with mammalian blood, is prepared by adding sufficient 6 per cent. aqueous sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ )



solution to 5.2 per cent. aqueous glucose solution to cause a sample of it to give a yellowish-green colour with the "Universal" Indicator of Messrs British Drug Houses. The amount of phosphate solution required depends on the length of time that the glucose solution has been made up. Since the vaginal fluid is sometimes acid, sometimes neutral and sometimes alkaline, it was thought best to use a neutral fluid. Since the vaginal fluid is a very complicated and variable one, it was obviously impossible to represent its chemical composition accurately in a laboratory investigation.

(9) 0.5 c.c. of this artificial vaginal fluid is transferred with a graduated pipette to each of two test-tubes in the damp chamber, and is left to warm up. One tube is the control tube, the other the experimental tube.

(10) 1 c.c. of semen is transferred with a graduated pipette to each of two other tubes in the damp chamber in the thermostat, and left to warm up. If the semen is stringy, it is difficult to measure 1 c.c. accurately when cold. Under these circumstances it is best to warm the semen slightly before transferring it to the tubes in the damp chamber.

(11) A quarter of an hour later, one-fifth of a pessary of the contraceptive to be investigated is placed in the experimental tube. If necessary it is pushed below the surface of the fluid.

(12) A quarter of an hour later again, the semen from one of the two tubes containing it is transferred with a pipette to the control tube.

(13) Air is bubbled through the contents of the control tube with a pipette. This mixes the fluids and promotes respiration by the sperms.

(14) The contents of the other tube of semen are transferred to the experimental tube.

(15) Air is bubbled through the experimental tube. If the fragment of pessary is caught in the froth, it is pushed down into the fluid again.

(16) The time is recorded as the start of the experiment.

(17) Two hollow-ground microscopical slides are labelled with a grease-pencil. One is labelled *C* (control), the other with letters denoting the name of the pessary under investigation.

(18) Over the grease-pencil lettering on each slide is fixed a blank gummed-paper label, stuck down at one side in such a way that it may be turned back to disclose the grease-pencil lettering. The object of this arrangement is explained in section 24.

(19) The labelled slides are placed in the thermostat.

(20) Ten minutes after the start of the experiment, air is again bubbled through each tube. A clean pipette is of course used for each tube. The repeated bubbling of air through the fluids not only promotes respiration, but also ensures that all the sperms present are acted upon by the spermicide at the same concentration. This is important, for only three drops are examined under the microscope, and inconclusive results would be obtained if it were not certain that the sperms in these three drops were representative of all the sperms in the tube.

(21) Twenty-five minutes after the start of the experiment (section 16), air is again bubbled through the control tube, and with the same pipette three drops of the fluid are transferred to the hollow of the slide marked C. (If the fluid does not form readily into drops, an equivalent amount is transferred.)

(22) A coverslip is applied. The slide is left on the floor of the thermostat. Three drops of fluid do not fill the hollow of the slide. A large bubble of air is included below the coverslip, which prevents the sperms from becoming inactive quickly from inability to respire.

(23) The process described in the last section is applied to the contents of the experimental tube and to the slide labelled with the name of the pessary under investigation.

(24) The slides are shuffled together, till the observer does not know which is which. Bias, conscious or unconscious, is thus avoided. The shuffling is particularly valuable when two or three different pessaries are being tested at the same time. (See section 30.)

(25) As exactly as possible half an hour after the start of the experiment (section 16), the slides are removed one by one from the thermostat and examined under the microscope with a  $\frac{1}{8}$  in. objective and No. 2 eyepiece, on the hot stage mentioned in section 2. The sperms in the peripheral part of the hollow of the slide are observed. If the sperms in the deep part of the hollow of the slide were examined, a different impression of the percentage active would be likely to be gained. Living human sperms have a marked tendency to apply themselves to surfaces. One may focus a long way up and down in the region of the deep part of the hollow without seeing many active sperms, while crowds of active ones are present in the shallow peripheral part.

(26) The activity of the sperms is recorded on the blank labels on the slides, in accordance with the system of grading which is explained at great length in the second paper. Here it must suffice to say that III indicates that the majority of the sperms are moderately active, II+ indicates that it cannot quickly be decided whether the majority are moderately active or not, and 0 indicates that not a single active sperm was seen in ten microscopical fields of view, while I, I+ and II indicate intermediate degrees of activity.

(27) The blank labels are turned aside to disclose the identity of the slides.

(28) If the control sperms show an activity of II+ or more, the result of the experiment is recorded.

(29) If the control sperms show an activity of less than II+, the result of the experiment is not recorded. It would have been more satisfactory if only those experiments could have been recorded in which the activity of the control sperms was III or III+. Unfortunately this was impossible, as the experiment could not be performed immediately after the ejaculation of semen. Indeed, nearly half of the samples of semen used came through the post.

(30) From one to three contraceptives may be tested at the same time against the same control.

(31) If the pessary forms a sediment which renders it impossible to observe the sperms properly under the microscope, or if it is so small or crumbly that one-fifth of a pessary cannot be separated in one piece, modifications of the technique are employed, which are described below.

(32) If the contraceptive under investigation is not sufficiently spermicidal to reduce the activity of the sperms to 0, the experiment described above is performed four or five times (not counting experiments in which the activity of the control sperms is less than II+).

(33) If the contraceptive under investigation is sufficiently spermicidal to reduce the activity of the sperms to 0 in four consecutive experiments, its efficiency is further tested by carrying out the experiment four or five times, using one-fiftieth of a pessary instead of one-fifth. When one-fiftieth of a pessary is used, the sediment is never great enough to necessitate the use of the modified technique. When one-fiftieth of a pessary is used, the contraceptive is at  $S/10$  concentration.

*Modification of the technique for pessaries which form a sediment  
(semori, monsol, finil, speton).*

In this modification of the usual technique, the following are placed in the thermostat in addition to the objects mentioned in section 1 of the description of the usual technique:

One glass capsule of about 100 c.c. capacity, with a watch-glass, concave side upwards, for a cover. (A watch-glass is used as a cover, because the foam produced by some pessaries will displace a flat cover. The watch-glass may be weighted if necessary.) One glass funnel with filter-paper and glass receptacle.

The procedure is as usual in other respects up to and including section 8, and then as follows:

(i) 10 c.c. of neutralised glucose solution is placed in the glass capsule. It is left to warm up.

(ii) 0.5 c.c. of neutralised glucose solution is placed in a specimen tube in the damp chamber, and left to warm up. This is the control tube.

(iii) A quarter of an hour after the 10 c.c. of glucose solution were placed in the capsule, 1 c.c. of semen is transferred with a graduated pipette to each of two empty specimen tubes in the damp chamber, and left to warm up.

(iv) Four whole pessaries of the contraceptive under investigation are thrown into the 10 c.c. of glucose solution in the capsule. The capsule is shaken. Four pessaries to 10 c.c. are equivalent to one pessary to 2.5 c.c., which is postulated as being the amount of fluid normally present in the vagina before ejaculation of semen. Much more fluid is taken than will be used, because in the subsequent filtration only a small part of it generally manages to pass the filter. Most is retained in the form of foam. (In the case

of finil, eight pessaries are thrown into the capsule instead of four, because two pessaries are directed to be used together at each coition.)

(v) Five minutes later the capsule is shaken again.

(vi) Ten minutes later, when the pessaries have been a quarter of an hour in the fluid, the capsule is shaken again, and the fluid filtered in the warm filter.

(vii) 0.5 c.c. of the filtrate is transferred with a graduated pipette to a tube in the damp chamber. This is the experimental tube. The remainder of the filtrate is discarded.

(viii) The semen from one of the tubes in the damp chamber containing it is transferred with a pipette to the control tube.

(ix) The usual procedure is now adopted, beginning at section 13.

*Modification of the technique for the quinine urea hydrochloride pessary.*

The quinine urea hydrochloride pessary is so small and powdery that it is not possible to cut off a piece of it exactly one-fifth of the total weight. Its spermicidal power is therefore tested at  $S$  concentration as though it were a pessary forming a sediment, since in that technique whole pessaries, not one-fifth pieces, are used. One pessary is dissolved in 2.5 c.c. of neutralised glucose solution at  $37^{\circ}\text{C}$ ., and 0.5 c.c. of this is transferred to the experimental tube. The fluid is not filtered, as complete solution takes place.

The same technique is used to test the efficiency of this pessary at  $S/10$  concentration, except that one pessary is dissolved in 25 c.c. of neutralised glucose solution. As before, 0.5 c.c. of this solution is transferred to the experimental tube.

This modification of the technique could be applied to any pessary in order to test it at very low concentrations. This would be necessary if any pessary were invented which always killed all sperms at  $S/10$  concentration. One would then need to test it at  $S/20$  or even  $S/100$ . This could easily be done by dissolving one pessary in 50 c.c. or 250 c.c. of neutralised glucose solution at  $37^{\circ}\text{C}$ ., and transferring 0.5 c.c. of the solution to the experimental tube.

TEST OF FOAMING PESSARIES IN THE ABSENCE OF THE FOAM-PRODUCING SUBSTANCES.

It was felt that foaming pessaries should be tested also without the foam-producing substances, for two reasons. Firstly, there is the possibility that carbon dioxide may only temporarily immobilise sperms. Secondly, if a pessary relies too largely on the carbon dioxide which it produces, it will not be effective unless placed in the vagina at precisely the proper time before the ejaculation of semen.

Guinea-pig sperms were used in this part of the work. Semori, finil and speton were tested, but not monsol. It did not seem worth while in the case of monsol, for this pessary is not yet on the market.

The experiments were carried out as follows.

The essential constituents of the pessary, other than the foam-producers, are dissolved in 0.9 per cent. sodium chloride solution, at the concentration at which they exist when one pessary is dissolved in 2.5 c.c., the postulated amount of vaginal fluid. Unessential constituents, such as French chalk, starch, lactose and egg-albumen, are omitted for the sake of simplicity. A sperm suspension is prepared by squeezing the tails of both epididymides of an adult male guinea-pig in 5 c.c. of B.G.S. (Instructions for the preparation of the fluid called B.G.S. are given in the second paper.) 1 c.c. of this is mixed with 0.5 c.c. of the fluid containing the essential constituents of the pessary, after both have been allowed to warm up for a quarter of an hour. The experiment is disregarded unless the control sperms show an activity of III or III+. In all other respects the experiment is carried out according to the usual technique for pessaries with human sperms.

Finil presents the difficulty that the essential constituents will not dissolve completely at the concentration of one pessary to 2.5 c.c. of 0.9 per cent. sodium chloride solution. It was not necessary, however, to invent a special technique for this pessary, for despite this handicap, it always killed all sperms.

The results were as follows:

Pessaries without foam-producers and unessentials, at *S* concentration.

Semori	0	0	0
Finil	0	0	0
Speton	III	III	III

This proves that, even without foam-producers, semori and finil kill all sperms at *S* concentration. Speton, on the contrary, is without effect if the foam-producers are omitted.

It was decided to test semori and finil at *S/10* concentration, without foam-producers. The experiments were carried out exactly as before, except that the essential constituents of the pessary, other than foam-producers, were dissolved in 0.9 per cent. sodium chloride solution at the concentration at which they exist when *one-tenth* of a pessary is dissolved in 2.5 c.c.

The control sperms showed an activity of III or III+ in each experiment.

The results were as follows:

Pessaries without foam-producers and unessentials, at *S/10* concentration.

Semori	0	0	0
Finil	I	I	I

This shows that both semori and finil are very spermicidal at *S/10* concentration even when the foam-producers are omitted. Semori has the advantage both here and in the standard experiment.

It was thought possible that the inefficiency of speton without foam-producers at *S* concentration might be due to the fact that sodium dichlorylsulphamidbenzoate (the only essential constituent other than foam-producers) is only effective when freshly dissolved. Accordingly the experiment was performed as before, except that the sodium dichlorylsulphamidbenzoate was

only dissolved at the last possible moment, 5 minutes before the start of the experiment (*i.e.* 5 minutes before section 16 in the standard technique). This substance dissolves almost immediately. Directly it had dissolved, 0.5 c.c. of the solution was transferred to the experimental tube. It had thus less than 5 minutes in which to warm up instead of a quarter of an hour, but this could not affect the result, as the 1 c.c. of sperm suspension was warmed for the usual quarter of an hour. The control sperms showed an activity of III or III+ in each experiment.

The result was as follows:

Pessary without foam-producers and unessentials, at <i>S</i> concentration, dissolved immediately before the experiment.		
Speton	III +	III

This proves that sodium dichlorylsulphamidbenzoate is not spermicidal even when freshly dissolved, and the spermicidal power of speton is wholly due to its foam-producers.

#### DISCUSSION.

It is clear that the cocoa-butter pessaries are less effective than the foam-producing ones and the quinine urea hydrochloride pessary, when tested by the techniques described in this paper. Chinosol, the most effective of the cocoa-butter pessaries, is shown by the standard experiments on human sperms to have the same spermicidal power as the least effective of the pessaries not containing cocoa-butter, namely finil.

There is some reason to think that cocoa-butter may have a mechanical effect in actual use, which renders it helpful rather than the reverse. This point is about to be studied under the auspices of the Birth Control Investigation Committee. Nevertheless these experiments show that cocoa-butter prevents the active substances (quinine or chinosol) from acting, in a way that can only be described as astounding. There is plenty of quinine or chinosol in a cocoa-butter pessary, but the cocoa-butter in some way prevents it from affecting sperms, even when the pessary has had 12 hours at the temperature of the body in which to melt and dissolve.

The great spermicidal power of the minute quinine urea hydrochloride pessary is noteworthy. The absence of any foam-producers in this pessary must nevertheless weigh against its use. It is usually placed on the upper (cervical) side of a rubber occlusive pessary.

The complete lack of spermicidal power in the supposedly essential constituent of speton is very remarkable. The pessary as a whole is quite effective, on account of the foam-producers. It is perhaps reasonable, however, to distrust a pessary which relies for spermicidal power wholly on its foam-producers.

Semori emerges from the test as the most effective pessary sold in England. It is effective with or without foam-producers.

## SUMMARY.

1. A technique is described for comparing the spermicidal powers of pessaries, using human sperms.
2. Those pessaries which do not contain cocoa-butter are more spermicidal than those that do.
3. Quinine and lactic acid pessaries, in cocoa-butter vehicles, are almost without effect upon sperms.
4. There is more than ten times as much quinine bisulphate in a quinine pessary as suffices to kill all sperms in half an hour, but the cocoa-butter prevents its action in some way which is not at present understood.
5. Semori is the most spermicidal pessary of the nine investigated. Even at one-tenth of the concentration at which it is normally used, it kills every sperm or nearly every sperm in half an hour.
6. Even if the foam-producing substances are omitted, semori remains effective.
7. The minute quinine urea hydrochloride pessary is nearly as effective as semori, but the absence of foam-producing substances in this pessary limits its usefulness.
8. Speton relies for its spermicidal power wholly upon its foam-producing substances. Its supposedly active substance, sodium dichlorylsulphamid-benzoate, is without effect upon sperms.

## REFERENCES.

- BAKER, J. R. (1930). The spermicidal powers of chemical contraceptives. I. Introduction, and experiments on guinea-pig sperms. *J. Hygiene*, **29**, 323-9.
- (1931). The spermicidal powers of chemical contraceptives. II. Pure substances. *Ibid.* **31**, 189-214.

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POSTSCRIPT. I have recently tested another foaming tablet, called "bircon," by the standard technique with guinea-pig sperms. This tablet weighs 0.64 gm. It consists of zinc sulphocarbolate, chinisol, starch, sodium bicarbonate and tartaric acid. It is made by Messrs Bircon Laboratories in London. The results were as follows:

At <i>S</i> concentration	0	0	0
At <i>S</i> /10 concentration	0	0	I

These results should be compared with those for other pessaries given on p. 312 of this paper. Bircon is seen to be more effective than the other pessaries tested: but no experiments have yet been performed to find to what extent it relies upon its foam-producers, nor has it been tested with human sperms.

## OBSERVATIONS ON *BACILLUS (HAEMOPHILUS) INFLUENZAE* WITH SPECIAL REFERENCE TO MORPHOLOGY AND COLONIAL CHARACTERS.

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(With Plates IV and V containing 21 figures.)

*Bacillus (Haemophilus) influenzae* is characterised by wide variation both in morphology of the individual organisms and in colonial type. The investigation here reported shows that such variation can be induced by altering the growth conditions, and that there is a considerable degree of correlation between individual morphological variation and colonial change. The variants obtained were examined further with respect to biochemical reactions, haemolysis and growth requirements; they were shown to retain the essential characteristics of the species.

### I. VARIATIONS IN MORPHOLOGY IN STRAINS OF *H. influenzae*.

Differences in morphological appearance form one of the characteristic features of those haemophilic bacilli which are commonly grouped together under the name "influenza bacillus." Typically Pfeiffer's bacillus is a short coccobacillus, but every gradation is met with between this coccobacillary type and the very long filaments or large thick curved forms of the atypical type. The colonial appearance also varies. Kristensen (1922) described large and small colonies, rough and smooth colonies, and colonies which were darker in colour than others. He stated that on suitable media the morphologically typical and atypical bacilli showed on the whole a corresponding difference in colonial form. The typical gave smooth colonies with a butyrous consistency, while the atypical tended to give rough colonies with a dry friable consistency. The dividing line between these two types however was not sharp. He also said that the type of colony retained its character relatively unchanged through a long period of cultivation. Wollstein (1915) drew attention to the readiness with which the coccobacillary type formed long threads in artificial cultures. Dible (1924), on the other hand, found that the morphology of a number of strains remained constant enough to enable one to recognise certain distinct types such as the coccobacillary type, and the bacillary and long filamentous types both of which he excluded from the true Pfeiffer group.



*Technique.*

All the strains of the influenza bacillus were isolated from nasopharyngeal swabs taken from normal people.

After isolation the usual procedure of studying variation was to grow the strain in about 100 c.c. of Fildes' broth (Fildes, 1920) in a small flask at 37° C. for several weeks. Cultures were made on a Fildes' plate about every other day and the colonies examined and filmed. It was usually only after 3 to 4 weeks' incubation that variants arose. These were picked off and cultured in tubes of Fildes' broth for 24 hours and again plated to see whether the variations remained constant. Stock cultures of the different variants were always kept in tubes of Fildes' broth at 37° C.

Other media were tried such as (1) Fildes' broth to which about 5 per cent. whole blood was added; (2) Fildes' broth to which about 5 per cent. of the homologous antiserum was added. The cultures to be examined were grown in the above media for varying lengths of time and plated every alternate day as above.

There seemed to be no advantage in the use of either of the above media over that of Fildes' broth alone.

Inoculation into mice was also utilised in the attempt to produce variants. In each case the mouse was inoculated intraperitoneally with 1 c.c. of a 24-hour culture in Fildes' broth; it was killed not later than the third day and films were made from the heart blood and peritoneal exudate, and cultures inoculated on to a Fildes' agar plate and into Fildes' broth which was again plated for variant colonies.

All films were stained by Gram's method, using as a counterstain 1/10 carbol fuchsin in distilled water applied for 15 minutes.

A description of the technique employed in the testing of sugar reactions, haemolysis, growth requirements, etc., is given in Section II under the biochemical characteristics.

*Definition of terms used.*

"Typical": Short coccobacilli,  $1\mu \times 0.8\mu$ . (Figs. 4 and 9.)

"Atypical, curly": Large curly bacilli, single or in short chains,  $10-50\mu \times 2\mu$ . (Fig. 17.)

"Atypical, coliform": Bacillary in form, similar in appearance to *Bact. coli communis*, but length varies from  $1.5\mu$  to  $8\mu$ . (Fig. 20.)

*Strain 8/7.*

This strain was first isolated from the nasopharynx on 19. iii. 29. It was then typical in morphology, showing very short bacilli and many coccobacilli; no long forms were observed. The colony after 24 hours on a Fildes' agar plate was smooth, dome-shaped and translucent, about 0.6 mm. in diameter with entire edge and butyrous consistency.

A culture was seeded into a small flask containing 100 c.c. of Fildes' broth and incubated at 37° C. Every alternate day one loop was plated on a Fildes' agar plate and the colonies and morphology examined. After 4 weeks' incubation there appeared on the plate two kinds of colony.

(1) A large smooth colony, 0.6 mm. in diameter after 24 hours, which on being filmed showed a typical morphology.

(2) A small colony, 0.1 mm. in diameter after 24 hours. This showed an atypical morphology with many long slender filaments, 40–50  $\mu$  in length.

Each type of colony was now picked off into Fildes' broth and its behaviour further examined.

The *large smooth* colony remained constant both with regard to morphology and colonial form. Repeated subcultures were made from Fildes' broth to Fildes' broth for long periods of time. (Figs. 1, 4.)

The *small* colony was very variable. It readily gave rise to two or three kinds of colonies:

*Large rough*, about 0.6 mm. in diameter, with a contoured surface and irregular edge. Films from this colony showed an atypical morphology with many very long curved filamentous forms, as well as shorter coliform bacilli. (Figs. 2, 5.) Where picked off into Fildes' broth and further subcultured it was found that the large rough colony always produced some small rough colonies, but very seldom gave rise to the large smooth type. On two occasions only was a large smooth type of colony obtained from a large rough culture.

(1) After repeated subculture of a large rough colony in Fildes' broth for over 14 days, some smooth colonies were obtained giving typical coccobacillary morphology. This result could not be repeated.

(2) An intraperitoneal inoculation into a mouse was made from a Fildes' broth culture of a large rough colony. The mouse died on the second day. Films from the peritoneal cavity showed filamentous gram-negative bacilli similar to those in the original inoculum, and on cultivation rough colonies were obtained. On subculturing again from a rough culture and plating, large rough colonies and small smooth colonies were obtained, the former giving an atypical morphology, the latter a typical. On further subculture the rough culture became entirely smooth.

*Small rough* colonies, about 0.1 mm. in diameter, with rough surface and very irregular edge. The morphology was mixed, showing about two-thirds coliform and one-third long curved filaments. (Figs. 3, 6.) This type remained relatively constant, giving rise to a majority of small rough and a few large rough colonies.

There follows (p. 524) a diagrammatic scheme showing the variability of strain 8/7.

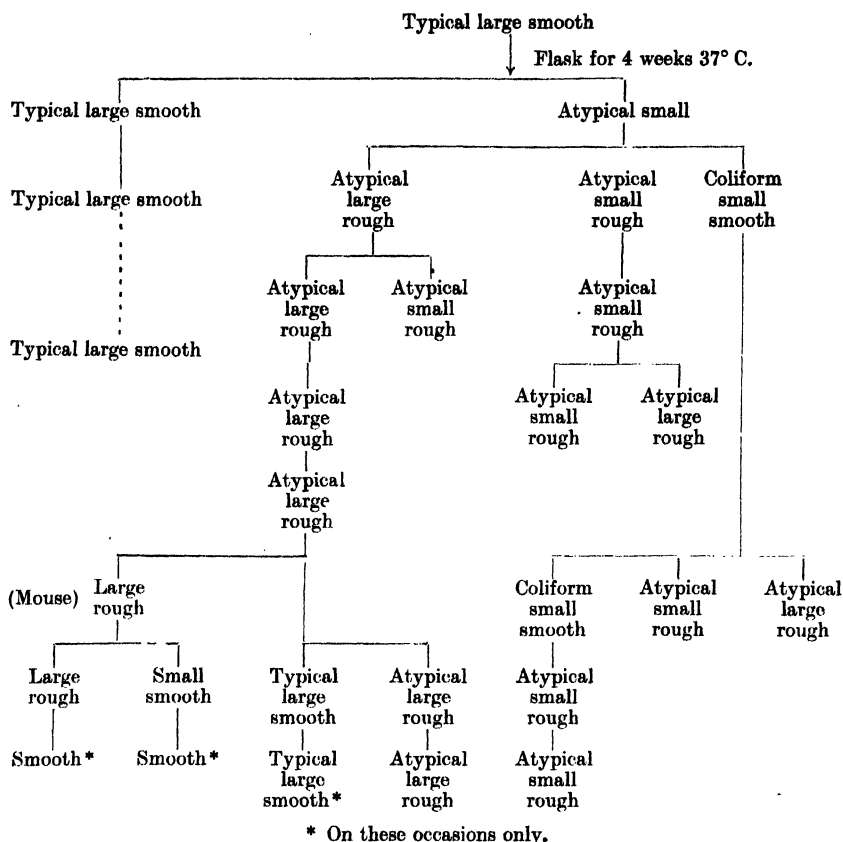
*Small smooth* colonies, about 0.1 mm. in diameter, with smooth surface and entire edge. These colonies were relatively few in number. The morphology was similar to that of the small rough. It is often difficult to distinguish this type of colony from the small rough. They are not very common. They have a coliform morphology and readily produce the small rough colony.

Thus in the case of this strain it was possible to demonstrate a change in the colonial form and morphology of a typical influenza bacillus into an atypical form by growing under certain conditions. Moreover, an atypical morphology was associated with a rough type of colony, whereas a typical morphology was associated with a smooth colony.

The rough and smooth strains were compared with regard to their biochemical characteristics, growth requirements, haemolysis, etc. The results are given below and show that there is no difference between any of the strains with regard to these characteristics.

	Dex- trose	Mal- tose	Man- nitolose	Lac- tose	Suc- rose	Sali- cin	In- dol	Haemo- lysis	Growth in		
									XV	V	X
Large smooth	A	0	0	0	0	0	+	0	++	0	0
Large rough	A	0	0	0	0	0	+	0	++	0	0
Small rough	A	0	0	0	0	0	+	0	++	0	0
Small smooth	A	0	0	0	0	0	+	0	++	0	0

For the technique employed in these tests see Section II.

*Bacillus influenzae**Strain 15/9.*

First isolated 15. v. 29 from nasopharynx. Typical coccobacillary in morphology. Colony smooth with entire edge and butyrous consistency, raised in centre, about 0.8 mm. in diameter.

This strain remained typical after repeated subculture in Fildes' broth tubes, on Fildes' or blood-agar plates, in flasks of Fildes' broth, or after passage through a mouse, for several months. Then after growing in a flask containing 100 c.c. Fildes' broth at 37° C. for 17 days, colonies of two different sizes appeared when plated on a Fildes' agar plate. These two colonies differed in their morphology as well as in their size.

(1) *Large colony*: similar to the original, smooth, 0.8 mm. in diameter with a typical morphology.

(2) *Small colony*: about 0.2 mm. in diameter with an atypical coliform morphology.

A second flask of Fildes' broth was taken and seeded with the original typical smooth and grown at 37° C. for 17 days and then plated. Three types of colony were observed.

(1) *Large smooth*: similar to original, 0.8–0.9 mm. in diameter, smooth surface, entire edge, butyrous consistency. Morphology typical, short coccobacilli. (Figs. 7, 9.)

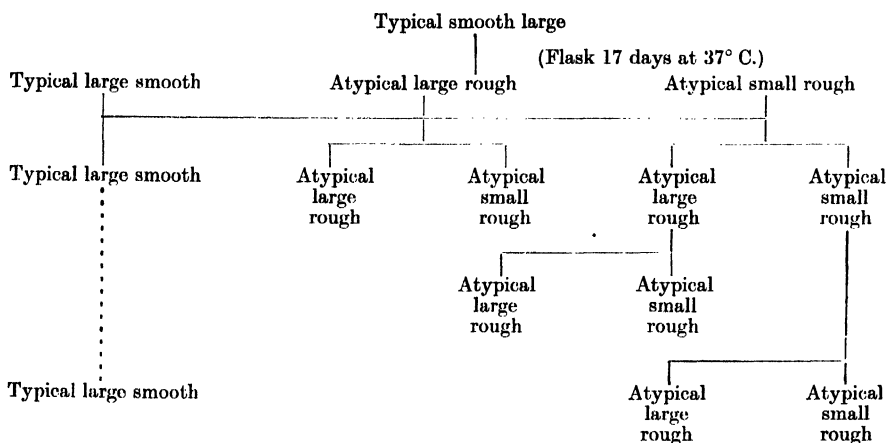
(2) *Large rough*: about 0.8 mm. in diameter, rough surface and irregular margin. Morphology atypical, coliform and large thick curved bacilli about 20–30  $\mu$  in length. (Figs. 8, 10.)

(3) *Small rough*: 0.2 mm. in diameter, rough surface and irregular outline. Morphology long coliform. (Fig. 11.)

Cultures were taken from each of the above three types of colonies into Fildes' broth and further observations were made as to their variability. It was found that the large smooth colony remained constant, always giving rise to similar colonies with a typical morphology. The large rough colony gave rise to colonies of different sizes, namely, the large rough and the small rough, both atypical in morphology.

The small rough colony behaved like the large rough giving rise to rough colonies both large and small.

A rough colony whether large or small was never observed to give rise to a smooth colony.



Here, as in strain 8/7, prolonged growth in Fildes' broth tended to produce rough colonies which had an atypical morphology.

With regard to biochemical characters the smooth and rough strains appeared to behave in a similar manner.

	Dex- trose	Mal- tose	Man- nitol	Lac- tose	Suc- rose	Sal- icin	In- dol	Haemo- lysis	Growth in		
	A	0	0	0	0	0	+	0	XV	V	X
Large smooth	A	0	0	0	0	0	+	0	+	0	0
Large rough	A	0	0	0	0	0	+	0	++	0	0

In the three strains described below, although there was a sharp difference in morphology, the only colonial difference noticeable was that of size.

#### *Strain 23/6.*

Isolated 4. iii. 29 from nasopharynx. Morphology typical coccobacillary.

Colonial form—smooth, 0.8 mm. in diameter, entire edge, raised centre, butyrous consistency.

This strain was kept in cultivation for some weeks and grown repeatedly in flasks of Fildes' broth. Finally, 6 months after isolation, on plating from a 3 weeks' culture, two kinds of colony were obtained.

(1) *Large smooth*: 0.8 mm. in diameter.

(2) *Small smooth*, 0.1 mm.–0.2 mm. in diameter.

The morphology of both types of colony was similar, namely, typical coccobacillary.

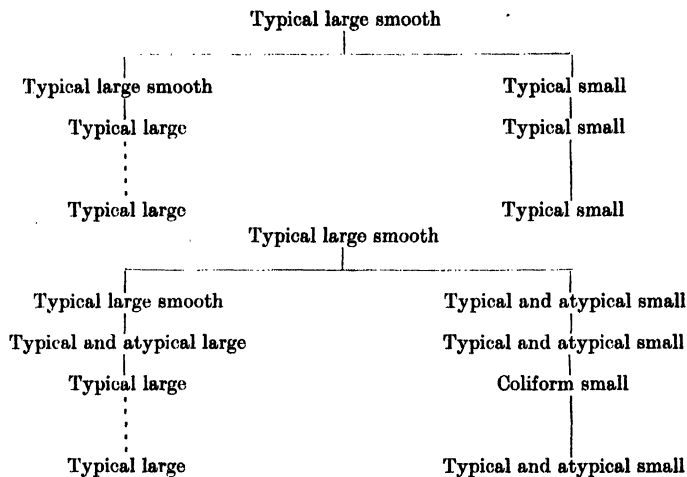
Each of the above colonies on subculturing remained constant, the large giving rise to large colonies, the small to small colonies.

Another flask was taken and seeded from the original strain. Plates were made at various intervals and again two types of colonies, large and small, were obtained, but this time the morphology differed. The large colonies still gave a typical morphology, but the

small colonies gave a mixture of small coccobacilli and very long thick curly forms. (Figs. 12, 13, 14.)

Further cultures from each of the above colonies showed great variability in morphology. The large smooth colonies generally showed an entirely coccobacillary morphology, but sometimes a few large thick bacilli were seen among the majority of small forms. The small colonies usually showed a mixed morphology with large numbers of thick curly forms, but occasionally the coliform type of morphology predominated, with only a few large bacilli.

The original strain was also grown in peptone water, to which 5 per cent. of an anti-serum for a typical strain was added. No difference in colonial form or morphology was observed.



The biochemical characters of the large and small colonies are as follows:

	Dex- trose	Mal- tose	Man- nite	Lac- tose	Suc- rose	Sal- cin	Indol	Haemo- lysis	Growth in		
									XV	V	X
Large	A	0	0	0	0	0	+	0	++	0	0
Small	A	0	0	0	0	0	+	0	+	0	0

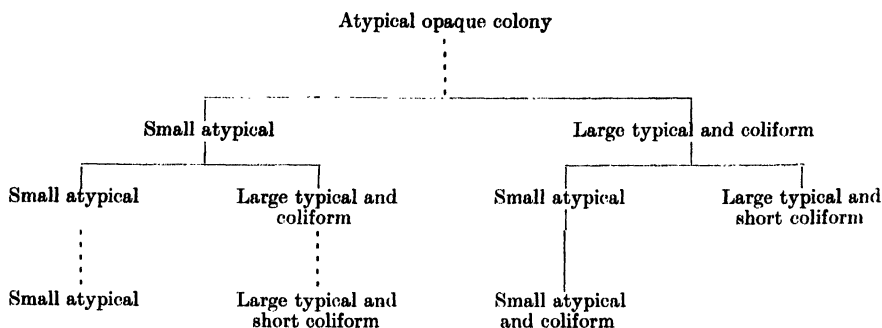
In this strain 23/6 a change from a typical to an atypical was observed with a difference in size of the colonial forms. Up to the time of writing, however, although the atypical small colony contained a preponderance of atypical forms, there were still some coccobacillary forms present, so that the change was not complete.

#### *Strain 24/10.*

Isolated 12. vi. 29. This strain was atypical when first isolated. The colonies were opaque, whitish in colour, and about 0.5 mm. in diameter. The morphology consisted chiefly of large curved forms and a few short coliform organisms.

After several subcultures large and small colonies were seen on a Fildes' agar plate (Fig. 15). Both kinds of colonies were translucent, slightly raised in the centre, and 0.8 mm. and 0.2 mm. respectively in diameter. A film made from a large colony showed very short coliform and coccobacilli (Fig. 16), while a film made from a small colony showed only large thick curly forms (Fig. 17). Cultures from both kinds of colony were picked off into Fildes' broth and remained on the whole fairly constant, although there was a tendency for the large colonies to show coliform and even a few long bacilli among the short forms (Fig. 18), and for the small colonies to show a few short forms.

Growth in Fildes' media to which 5 per cent. whole blood had been added brought out the differences between typical and atypical morphology more clearly.



Biochemical characters are as follows:

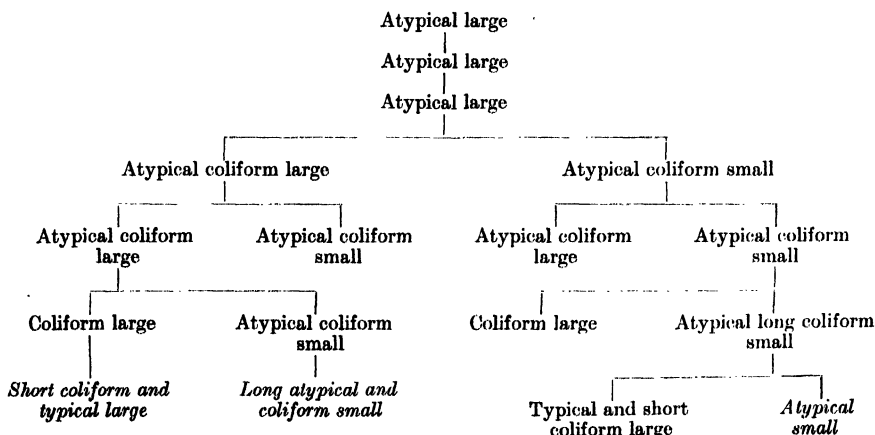
	Dex- trose	Mal- tose	Man- nite	Lac- tose	Suc- rose	Sali- cin	Indol	Haemo- lysis	Growth in		
									XV	V	X
Large	A	0	0	0	0	0	0	0	+	0	0
Small	A	0	0	0	0	0	+	0	+	0	0

Here the change was from an atypical to a typical with corresponding changes in colonial form. The difference in indol production between the two types should be noted.

*Strain 4/5.*

Isolated February 1929. Atypical, long slender bacilli  $4-5\mu \times 0.5\mu$ , stained faintly. Colony translucent, butyrous, raised in centre, 0.8 mm. in diameter.

Repeated cultivation (subculturing every week) in Fildes' broth gave no indication of variation for many months. In September, however (7 months after isolation), small colonies about 0.1-0.2 mm. in diameter were observed among the larger colonies (Fig. 19). The morphology appeared to be very similar in both types, coliform with many long slender forms and some shorter ones. After cultivation of the two kinds of colony for some weeks a much sharper difference in morphology could be seen. The large colonies now consisted of short organisms (Fig. 20), while the small colonies were almost entirely composed of long slender bacilli some  $50-70\mu$  long. (Fig. 21.)



Biochemical characters are as follows:

	Dex- trose	Mal- tose	Man- nite	Lac- tose	Suc- rose	Sali- cin	Indol	Haemo- lysis	Growth in		
									XV	V	X
Large	A	0	0	0	0	0	+	0	++	0	0
Small	A	0	0	0	0	0	+	0	++	0	0

Summarising the colonial and morphological variations among the strains studied, there seems to be some association between morphology and colonial form. Organisms having a typical morphology seem on the whole to produce a smooth colony, while an atypical morphology seems to be either associated with a rough colony, or with one very much smaller in size.

It seems possible, moreover, to produce rough variants with atypical morphology from a typical smooth strain. Smooth typical variants can also (although it is less common) be obtained from rough atypical strains.

The biochemical characters remained, on the whole, unchanged. One exception is seen in the change from a negative indol to a positive.

In view of these results it does not seem justifiable, in the present state of our knowledge, to exclude from the species *H. influenzae* those haemophilic bacilli which possess an atypical morphology, but require both X and V factors for their growth, and fail to produce haemolysis. It is, of course, quite possible that this group will be finally subdivided, either into separate species or varieties on the basis of fermentation reactions, or in some other way. Since, however, the morphology and colonial appearances may undergo marked changes under prolonged cultivation in the laboratory, while the fermentation reactions and growth requirements remain constant, it seems clear that morphology alone cannot be accepted as an adequate criterion for systematic differentiation.

It does not, of course, follow that morphological differences are devoid of significance from the pathological point of view. There is, indeed, general agreement that the coccobacillary form is that usually isolated from cases of active infection in man, with the single exception of cases of influenzal meningitis; while a considerable proportion of the haemophilic bacilli recovered from the normal mouth or throat show an atypical morphology (see Flemming and Maclean, 1930). The meaning of these facts is, at the moment, quite obscure.

## II. THE BIOCHEMICAL CHARACTERS OF MORPHOLOGICALLY TYPICAL AND ATYPICAL STRAINS.

The biochemical reactions of the variants obtained during this investigation, and of the parent strains from which they were derived, have been set out in tabular form above. In addition to these, a large number of other strains have been studied from this point of view.

### (1) *Fermentation of sugars.*

Three types of media were tried; in each case Andrade's reagent was added as an indicator.

(a) Peptone water sugars, to which 5 per cent. of Fildes' tryptic blood

digest was added. In this medium a copious growth was obtained but it had the disadvantage of having a faint reddish tinge, and thus slight traces of acid were obscured. Control tubes were always put up, and it was usually easy to detect any difference in colour between the tube fermented and the control tube. However, it was decided to discard the medium owing to this difficulty.

(b) Peptone water sugars to which an extract of boiled rabbits' blood was added (Stillman and Bourne, 1920). Here slight traces of acid could be easily seen, but fermentation did not always occur as readily as in the third method.

(c) Peptone water sugars containing yeast extract and haematin. To each tube containing approximately 5 c.c. of sugar medium about 0.1 c.c. of yeast extract and one small loop of a 1 per cent. alkaline solution of haematin were added. This last medium proved an excellent one for testing fermentation reactions. Not only was any acid produced perfectly obvious, but the results remained on the whole constant.

Table I. *Showing the percentages of the strains examined fermenting the various test substances.*

	Dex- trose	Mal- tose	Suc- rose	Man- nite	Lac- tose	Sal- icin	No sugars fer- mented	No.
Typical	21 44.8 %	9 19.1 %	2 4.3 %	— —	— —	— —	21 44.8 %	47 —
All atypicals	51 54.8 %	24 25.8 %	21 22.6 %	1 1.1 %	6 6.5 %	2 2.2 %	32 34.4 %	93 —
Coliform	36 53.7 %	11 16.6 %	14 20.9 %	1 1.5 %	2 3.0 %	— —	25 37.3 %	67 —
Atypical long and wavy	15 57.7 %	13 50.0 %	7 26.9 %	— —	3 11.9 %	2 7.7 %	7 27.0 %	26 —
Total { Typical, atypical coliform	72 51.4 %	33 23.6 %	23 16.4 %	1 0.7 %	5 3.6 %	3 2.1 %	53 37.8 %	140 —

It will be seen from the above table that dextrose, maltose and sucrose are the sugars most often fermented, in fact among the "typicals" they are the only sugars to be fermented. Very small numbers of "atypicals" ferment lactose, mannite and salicin. Atypicals are definitely more active than the typicals. This is also evidenced by the lower percentage of atypicals which ferment no sugars. The coliform, as distinct from the atypical, are less active than the real atypicals, although they are more active in most cases than the typicals. These results are in general agreement with those recorded by other workers (Levinthal, 1918; Stillman and Bourne, 1920; Kristensen, 1922; Dible, 1924).

## (2) *Production of indol.*

For the testing of indol, duplicate tubes were always put up, one of Fildes' broth, the other of peptone water, to which were added yeast extract and haematin. The tubes were incubated at 37° C. for 5 days, then a layer of ether was added to each tube and about  $\frac{1}{2}$  c.c. of paradimethyl-aminobenzaldehyde and  $\frac{1}{2}$  c.c. of potassium persulphate. There was never any doubt as to the



production of a red colour in the positive cases. The Fildes' media proved to be slightly better for the demonstration of this reaction, possibly owing to the better growth obtained in it.

Table II. *Production of indol.*

Typicals		Coliform		Atypicals	
Indol +	Indol -	Indol +	Indol -	Indol +	Indol -
46	26	43	49	11	50
63.9 %	36.1 %	46.7 %	53.3 %	18.0 %	82.0 %

Among the typicals two-thirds are indol positive and one-third are indol negative. In the true atypicals (excluding coliform) 82 per cent. are indol negative. The coliform are intermediate between the typicals and the atypicals, with just over one-half indol negative.

### (3) *Reduction of nitrates and action on litmus milk.*

Some strains were tested for the reduction of nitrates. All produced nitrites. Litmus milk was unchanged in every case.

*Haemolysis.* All the strains described in this paper were non-haemolytic. Any organism isolated from the nasopharynx which produced haemolysis was discarded. Haemolysis was tested in two ways:

(1) On a whole blood-agar plate, where any clearing or discoloration round the colonies was taken as an indication of haemolysis.

(2) In test-tubes of peptone water to which X and V factors had been added. After growth had occurred in this medium for 24 hours at 37° C., 1 c.c. of a 5 per cent. suspension of washed rabbit red cells was added and the mixture further incubated at 37° C. for another hour, then placed at 0° C. overnight. Rabbits' cells were found to be far better than either sheep or horse blood.

### *The symbiosis phenomenon.*

All the strains showed this phenomenon (Grassberger, 1897) in varying degrees. A 1 per cent. whole blood-agar plate was used, spread with the strain, and then inoculated with a few isolated streaks or patches of *Staphylococcus albus*. An abundant growth occurred round the *Staphylococcus*.

*Growth requirements.* The list of workers on the growth-requirements of Pfeiffer's bacillus is a large one (Ghon and Preyss, 1904; Davis, 1917; Olsen, 1920; Fildes, 1920, 1921, 1922, 1923; Thjötta and Avery, 1921; Kristensen, 1922; to mention a few). All agree that the bacillus is dependent on a substance X, present in red blood cells, active in minute amounts, and thermostable. There is also required a second, vitamin-like factor, V, found in yeast, and certain animal and vegetable tissues.

Most of the strains studied in this work were tested with regard to their growth requirements. Three tubes of peptone water were taken, each containing 5 c.c. To the first a small loop of a 1 per cent. alkaline solution of haematin

was added, to the second about 0.1 of a yeast extract prepared according to Thjötta and Avery (1921), to the third both haematin and yeast extract were added. Thus the first tube contained the *X* factor only, the second tube contained the *V* factor only, whereas the third tube contained both *X* and *V* factors.

A 24-hour Fildes' plate of the strain of influenza bacillus to be tested was taken. A small amount of growth was emulsified in sterile saline to give an opacity of about  $500 \times 10^6$  per c.c. Dilutions were then made, using tubes containing 10 c.c. sterile water and sterile dropping pipettes graduated so as to deliver fifty drops to the c.c. A fresh sterile pipette was used for each dilution. A final dilution of 50,000 organisms per c.c. was obtained. Sufficient of this dilution was then added to each of the above three tubes to give from 200 to 1000 organisms per c.c. in the tubes to be tested.

Table III. *Growth in media containing X and V.*

Strain	Amount added to 5 c.c. peptone water of a suspension con- taining 50,000 organisms per c.c.		<i>X</i>	<i>V</i> *	<i>XV</i>
	(drops)				
A <sub>1</sub>	3	0	0	0	+
A <sub>1</sub> (i)	3	0	0	0	+
A <sub>2</sub>	1	0	+	+	+
291	3	0	0	0	+
I 1	1	0	0	0	+
LPE	3	0	0	0	+
P <sub>2</sub>	5	0	0	0	+
RIN	3	0	0	0	+
RIN <sub>2</sub>	1	0	+	+	+
Ry	1	0	0	0	+
	2	0	+	+	+
RINP	3	0	0	0	+
13	3	0	0	0	+

\* Batch D of yeast extract (see Table IV) was not used here.

The method of preparing the yeast extract was the same in the case of each of the batches used (Thjötta and Avery, 1921). One batch of extract (D, see Table IV) behaved very differently from the others, in that it allowed the growth of all strains tested without the addition of haematin. Moreover, the organisms could be sub-cultured indefinitely in media containing this extract without the addition of the *X* factor. It is probable that this extract actually contained an amount of the *X* factor sufficient for the growth requirements of *H. influenzae*. The results obtained with it must, of course, be disregarded; but it affords a useful illustration of the dangers of an undue reliance on any particular yeast extract as a source of the *V* factor for testing purposes. Growth in any tube was decided not only on the presence of turbidity or deposit, but always in addition by plating on to a Fildes' plate. Thus small amounts of growth could be observed which would otherwise be missed if opacity alone were taken as the criterion of growth.

In Table III it is seen that in the case of strains A<sub>2</sub> and RIN<sub>2</sub>, although only 1 drop of the saline dilution was added (giving 200 organisms per c.c. in the

Table IV. *Showing growth in V factor alone.*

Strain	Amount added to 5 c.c. peptone water of suspension containing 50,000 organisms per c.c. drops	Yeast batch																								
		D										E										F				
		1st testing					2nd testing					Successive subculturing										1st testing 2nd testing 1st testing				
		XV	V	X	XV	V	X	V <sub>1</sub> 1 loop	V <sub>2</sub>	V <sub>3</sub>	V <sub>4</sub>	V <sub>5</sub>	V <sub>6</sub>	V <sub>7</sub>	V <sub>8</sub>	V <sub>9</sub>	V <sub>10</sub>	XV	V	X	XV	V	X	XV	V	X
23/6	1	++	++	0	++	++	++	++	++	++	++	++	++	++	++	++	++	+	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0
20/4	1	++	++	0	++	++	++	++	++	++	++	++	++	++	++	++	++	+	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0
8/7	1	++	++	0	.	.	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0
11/3	1	++	++	0	++	++	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.	.
8/4	1	++	++	0	.	.	++	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0
4/5	1	++	++	0	.	.	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.	.
11/6	1	++	++	0	.	.	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.	.
9/7	1	++	++	0	.	.	++	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0

tubes tested), growth occurred in the tube containing *V* alone. In the case of Ry, 1 drop gave no growth in *V* alone, but 2 drops (400 organisms per c.c.) gave a definite growth. In all other cases 3 drops (600 organisms per c.c.) gave no growth.

Fildes (1923) added 0.05 c.c. of a suspension containing 40,000 organisms per c.c. to 10 c.c. peptone water, thus giving 200 organisms per c.c. in the tested tubes.

It seems unlikely therefore that, in the above two cases, growth in *V* alone is due to minute traces of the *X* factor carried over from the Fildes' plate, since in most other cases larger amounts gave no growth.

With regard to the growth requirements of the influenza bacillus, by far the greater number require both an *X* and a *V* factor. In a few cases, however, it appears from the above results that organisms which in every other respect would be undoubtedly classified as true *H. influenzae* will grow in *V* alone, although even in these cases they will not grow in all batches of yeast extract.

#### SUMMARY.

1. Several strains of the influenza bacillus were grown under varying conditions for many months in order to ascertain whether morphology and colonial appearances could be permanently modified.

2. It was found that a modification in morphology occurred in all the strains studied.

3. Typical coccobacillary forms were changed to atypical long-curved forms.

4. The reverse change from atypical was also observed but was not so frequent, nor, when it occurred, was it so constant.

5. The change took place most readily after growth in a flask of Fildes' broth for several weeks or months at 37° C.

6. There was found to be a definite association between morphology and colonial form. The typical strains produced smooth colonies, while the atypical gave rough colonies, or colonies very much smaller than those produced by the typical.

7. When the change in morphology took place the change in colonial form occurred at the same time.

8. The biochemical characters remained on the whole unchanged.

9. One hundred and forty strains were examined for their fermentation reactions. The sugars most frequently fermented were dextrose, maltose and sucrose.

10. The atypicals were more active fermenters than the typicals.

11. 63.9 per cent. of the typicals, and 18 per cent. of the atypicals, gave a positive indol reaction.

12. Although the ability to grow on *X* or *V* alone affords an important differential criterion within this group, great care is necessary in interpreting

the results obtained, especially in the case of those strains which grow on *V* in the absence of *X*. Different yeast preparations may give very different results in this respect.

13. All the strains tested were non-haemolytic.

I wish to express my indebtedness to Prof. Topley for his advice and help throughout this investigation.

## EXPLANATION OF PLATES IV. AND V.

### PLATE IV.

*Bacillus (Haemophilus) influenzae*. Colonies and film preparations.

Colonies were grown on Fildes' agar plates for 24 hours at 37° C. Films were stained by Gram's method and counterstained with a 1 in 10 dilution of carbol fuchsin.

- Fig. 1. Large smooth colony of strain 8/7. ( $\times 8$ .)
- Fig. 2. Large rough colony of strain 8/7. ( $\times 8$ .)
- Fig. 3. Small rough colony of strain 8/7. ( $\times 8$ .)
- Fig. 4. Film preparation from a large smooth colony of strain 8/7. ( $\times 1500$ .)
- Fig. 5. Film preparation from a large rough colony of strain 8/7. ( $\times 1500$ .)
- Fig. 6. Film preparation from a small rough colony of strain 8/7. ( $\times 1500$ .)
- Fig. 7. Large smooth colony of strain 15/9. ( $\times 8$ .)
- Fig. 8. Large rough colony of strain 15/9. ( $\times 8$ .)
- Fig. 9. Film preparation from a large smooth colony of strain 15/9. ( $\times 1000$ .)
- Fig. 10. Film preparation from a large rough colony of strain 15/9. ( $\times 1000$ .)
- Fig. 11. Film preparation from a small rough colony of strain 15/9. ( $\times 1000$ .)

### PLATE V.

- Fig. 12. Large and small colonies of strain 23/6. ( $\times 8$ .)
- Fig. 13. Film preparation from a large colony of strain 23/6. ( $\times 1000$ .)
- Fig. 14. Film preparation from a small colony of strain 23/6. ( $\times 1000$ .)
- Fig. 15. Large and small colonies of strain 24/10. ( $\times 8$ .)
- Fig. 16. Film preparation from a large colony of strain 24/10. ( $\times 1000$ .)
- Fig. 17. Film preparation from a small colony of strain 24/10. ( $\times 1000$ .)
- Fig. 18. Film preparation from a large colony of strain 24/10 showing a mixture of long and short forms. ( $\times 1000$ .)
- Fig. 19. Large and small colonies of strain 4/5. ( $\times 8$ .)
- Fig. 20. Film preparation from a large colony of strain 4/5. ( $\times 1000$ .)
- Fig. 21. Film preparation from a small colony of strain 4/5. ( $\times 1000$ .)





## REFERENCES.

- DAVIS, D. J. (1917). *J. Inf. Dis.* **21**, 392.  
— (1921). *Ibid.* **29**, 178, 187.  
DIBLE, H. (1924). *J. Path. and Bact.* **27**, 151.  
FILDES, P. (1920). *Brit. J. Exp. Path.* **1**, 129.  
— (1921). *Ibid.* **2**, 16.  
— (1922). *Ibid.* **3**, 210.  
— (1923). *Ibid.* **4**, 265.  
— (1924). *Ibid.* **5**, 69.  
FLEMMING, A. and MACLEAN, I. H. (1930). *Ibid.* **11**, 127.  
GRASSBERGER, R. (1897). *Z. f. Hyg.* **25**, 453.  
KRISTENSEN, M. (1922). *Haemoglobinophilic Bacteria*. (Copenhagen.)  
LEVINTHAL, W. (1918). *Z. f. Hyg.* **86**, 1.  
STILLMAN, E. G. and BOURNE, JANET (1920). *J. Exp. Med.* **32**, 665.  
THJÖTTA, T. and AVERY, O. T. (1921 *a*). *Ibid.* **34**, 97.  
— — (1921 *b*). *Ibid.* **34**, 455.  
WOLLSTEIN, MARTHA (1915). *Ibid.* **22**, 445.

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## ON THE STATISTICAL MEASURE OF INFECTIOUSNESS.

BY M. GREENWOOD, F.R.S.

WE all recognise that some diseases are more "catching" than others. Every mother knows that measles is very catching and most people set aside a group of common complaints, measles, mumps, whoóping-cough, scarlet fever, diphtheria—perhaps roughly in that order—as catching complaints. Then again, still keeping ourselves within the circle of ideas of educated non-medical people, one has such complaints as common colds or influenza which one thinks of as running through a house indeed but does not put quite into the measles category, as one feels that factors determine the spread other than mere proximity to a sick person. Lastly, one has some illnesses, gonorrhoea would be a fair example, which everybody recognises to be spread wholly by contagion, almost always by a particular method of contagion, but does not regard as catching at all in the sense that measles and whooping-cough are catching. When we enquire into the reasons of these opinions they will be found, I think, to be these.

An illness is held to be catching when it has usually been possible to explain the existence of a case of it by close association (of some kind) with an immediately pre-existing case; the notion of more or less infectiousness depends upon some appraisalment of the proportion of persons attacked to persons exposed to the risk of attack. Mothers observe that when a child sickens with measles most of the other children take the complaint within a period of days or weeks, when a child sickens with scarlet fever the proportion of others attacked is smaller, and so on.

When we are dealing practically with such complaints as measles, rough criteria such as these are sufficient; but when we seek a deeper knowledge of the epidemiology even of these common complaints they are inadequate, and when we pass to the debatable region of diseases of which the infectiousness is quite uncertain they are altogether useless. Epidemiologists without any predilections for statistical analysis have, therefore, been forced to give these popular notions an at least quasi-statistical form. They have studied the distribution of "multiple" cases of disease in groups—families, inmates of houses, etc.—and sought to draw conclusions from the frequency distribution. If, they have argued, a "case" does not breed other "cases" in the exposed to risk, then if a number of groups have been observed through a finite interval, we shall find but few instances in which any one group contains more than one affected member, and in those few cases we shall be able to account for the independent origin—independent one of another—of the multiple cases. If, on the other hand, one case does breed others, the more intense the effect the

more closely will the distribution of groups tend to approximate to the opposite extreme, viz.  $n$  cases in a group of  $n$  persons. The criterion of infectiousness is the degree of approximation to one or other extreme  $J$ -shaped distribution, only a single case in each group of  $n$ ,  $n$  cases in each such group. Thus the fact that in 8635 families each containing at least one member with poliomyelitis, 96 per cent. had *only* one affected member, has been used as an argument against the belief that personal contact is an important factor of dissemination of this complaint<sup>1</sup>.

The object of this paper is to examine the statistical problem in greater detail. There are two difficulties, one really insuperable, the other insuperable by me.

The generally insuperable difficulty (insuperable in the present state of knowledge) is that *accurate* data are scanty; a great majority of the published records of multiple cases omit essential particulars. It is evidently futile to apply elaborate arithmetical machinery to records of "multiple" cases which omit any appraisalment either of the characteristics (age, sex, previous history of exposure) or even of the numbers constituting the groups. In the rare instances when this information is afforded, the number of groups is tiny. Had it not been for the kindness of my friend Dr Stocks, this paper must have been wholly "in the air." The difficulty insuperable by me is that before one has given much time to the subject—I have worked at it, off and on, for years—one encounters mathematical difficulties of a serious nature, the conquest of which requires special knowledge and ability I do not possess.

These considerations are by way of preface to and apology for the report of an investigation practically and theoretically inadequate but, as I hope, suggestive.

The general problem is this.  $N$  households (families or groups defined in some precise way) have been observed over a period of time  $T$  and, at the close of observations,  $N_0$  of the groups have recorded no case of the disease,  $N_1$  have recorded 1 case,  $N_2$  2 cases, ...  $N_r$   $r$  cases; what light does the form of this distribution throw upon the aetiology of the cases? The problems to be discussed fall under two classes. In both the number of members of each group is supposed to be constant, but in one the number of cases within a group may, in the other it may not exceed the number of persons constituting the group. To the former class belong such problems as that of "cancer houses" where the number of cases may exceed the number of inhabitants at a given instant, since the record may cover some generations of inhabitants, or of the distribution of non-fatal accidents amongst employees, since one employee may sustain many accidents; to the latter the distribution of cases of measles, scarlet fever, etc., in families or houses when the period of observation  $T$  is short. The distinction is clear; in the two former cases, although not more than all the inhabitants can be sick of cancer at one and the same time, and a workman cannot have an unlimited number of accidents, however trivial, at precisely

<sup>1</sup> *Epidem. Report, Health Section, League of Nations*, March 15th, 1930, pp. 100-1.

the same instant of time, yet in the one case the period of observation  $T$  may be so long that successive generations of inmates have been exposed to risk, and in the other the period of actual happening is so short in comparison with  $T$  that there is no real impropriety in postulating an upper limit to the possible incidence of multiple cases far greater than the actual number of persons. But, when, as always happens in the testing of a hypothesis and sometimes happens in the framing of it, the observed number of cases, which I shall always denote by  $n$ , is taken to be, like  $N$ , an ultimate datum of observation, we must remember that the upper limit is really fixed. The scientific discussion of problems of the first class was begun in 1911-12 by Maynard and Troup and by Pearson. Pearson, discussing cancer houses, used the following argument. Supposing the distribution of cases in houses to be absolutely random, analogous to throwing balls at random into equally accessible pigeon holes, then the chance that any one case will fall in any one house is  $1/N$  and that it will not is  $(N - 1)/N$ , therefore the distribution of the whole  $n$  cases should be given by the terms of the binomial  $N [1/N + (N - 1)/N]^n$ . In comparing the observed distribution with that required by the hypothesis, we must not use the ordinary Goodness of Fit test, because not only  $N$  but also  $n$  is fixed; Pearson showed that in our comparison we must not include the frequency of houses with no cases. In 1920 Yule and I discussed the cognate problem of multiple accidents. We pointed out a *theoretical* objection to Pearson's treatment, viz. that when  $N$  and  $n$  were not large two sets of data, one recording  $n$  cases in  $N$  houses, the other  $kn$  cases in  $kN$  houses, would have different representative binomials which seemed objectionable. We suggested that a better analogy than that of throwing  $n$  balls in  $N$  pigeon holes might be to liken the exposed to risk to targets subjected to bombardment, the time of bombardment  $T$  to be divided into intervals so short that within each not more than one hit could be registered on a target. On that hypothesis the distribution of 0, 1, 2, etc., hits at the end of  $T$  should be given by the Poisson limit to the binomial with parameter  $n/N$ . The hypotheses are slightly different and, as I still think, the second is the more appropriate, but in practice  $N$  would usually be large and  $n/N$  always finite, so that there would be little arithmetical difference between the results. Both the pigeon-hole schema and the variant Yule and I favoured can be generalised (see Greenwood and Yule (1920), pp. 259, etc.), although only in the simplest case did we reach a form suitable for the deduction of the requisite parameters from the moments of the statistics. The generalisation of the pigeon-hole schema is of some interest, because it suggests a way of approach which, possibly, might repay further mathematical treatment.

When there is no bias, i.e. when the fact that a particular pigeon hole has already received one or more of the first  $r$  balls neither increases nor diminishes the probability that one or more of the remaining  $n - r$  balls shall fall into it, we can deduce the distribution, viz.  $N \left( \frac{1}{N} + \frac{N-1}{N} \right)^n$ , term by term, as follows. Suppose we use the symbol  $f_r$  to denote the number of pigeon holes

which after  $r$  balls have been distributed contain  $s$  apiece. For all values of  $r$ ,  
 $\sum_{s=0}^{s=n} {}_r f_s = N$ .

Let  $E_r f_s$  denote the mathematical expectation of  ${}_r f_s$ , i.e. the value to which the mean or average value of  ${}_r f_s$  will tend when deduced from a large number of observations or experiments. Then  $E_r f_s$  will be a constant dependent on  $N$ ,  $n$ ,  $r$  and  $s$ , and hence  $E(E_r f_s)$ , which we may write  $E^2 {}_r f_s = E_r f_s$ .

When  ${}_{r-1} f_0$  is known,  ${}_r f_0$  will differ from  ${}_{r-1} f_0$  by  $-1$  or  $0$  according as the  $r$ th ball falls into an unoccupied pigeon hole or not. The probability of the first event is  ${}_{r-1} f_0 / N$  and of the second  $1 - \frac{{}_{r-1} f_0}{N}$ . Hence the *mathematical expectation* of the difference

$${}_r f_s - {}_{r-1} f_s = (-1) \frac{{}_{r-1} f_0}{N} + (0) \left(1 - \frac{{}_{r-1} f_0}{N}\right),$$

or 
$$E({}_r f_0 - {}_{r-1} f_0) = -\frac{1}{N} {}_{r-1} f_0.$$

Hence 
$$E^2 {}_r f_0 = E^2 {}_{r-1} f_0 - \frac{1}{N} E {}_{r-1} f_0,$$

or 
$$E_r f_0 = \left(1 - \frac{1}{N}\right) E_{r-1} f_0.$$

But  ${}_0 f_0 = N$ ,  $\therefore E_0 f_0 = N$ .

Hence 
$$E_n f_0 = \left(1 - \frac{1}{N}\right)^n N = N \left(\frac{N-1}{N}\right)^n.$$

More generally, the  $r$ th ball can fall into one of the  ${}_{r-1} f_s$  pigeon holes which already contain  $s$  balls each. This gives

$${}_r f_s - {}_{r-1} f_s = -1 \quad \text{.....(i),}$$

or into one of the  ${}_{r-1} f_{s-1}$  pigeon holes which contain  $s-1$  balls each. This gives

$${}_r f_s - {}_{r-1} f_s = +1 \quad \text{.....(ii),}$$

or into one of the remaining  $N - {}_{r-1} f_s - {}_{r-1} f_{s-1}$  pigeon holes. This gives

$${}_r f_s - {}_{r-1} f_s = 0 \quad \text{.....(iii).}$$

The probabilities of the three events are

$${}_{r-1} f_s / N, \quad {}_{r-1} f_{s-1} / N, \quad 1 - \frac{{}_{r-1} f_s + {}_{r-1} f_{s-1}}{N}.$$

Hence 
$$E({}_r f_s - {}_{r-1} f_s) = -\frac{{}_{r-1} f_s}{N} + \frac{{}_{r-1} f_{s-1}}{N},$$

and noting again that

$$E^2 {}_r f_s = E_r f_s,$$

we have 
$$E_r f_s = \left(1 - \frac{1}{N}\right) E_{r-1} f_s + \frac{1}{N} E_{r-1} f_{s-1}.$$

The solution of this difference equation for  $E_r f_s$  is

$$E_n f_s = N \binom{n}{s} \frac{1}{N^s} \left(\frac{N-1}{N}\right)^{n-s}.$$

Let us suppose now that the chance of the  $r$ th ball falling into an empty pigeon hole differs from  $\frac{r-1}{N}f_0$ , so that the chance of falling into an occupied pigeon hole is not  $1 - \frac{r-1}{N}f_0$  but equal say to  $t \left(1 - \frac{r-1}{N}f_0\right)$ , so that the chance of falling into an empty pigeon hole becomes

$$1 - t \left(1 - \frac{r-1}{N}f_0\right) = 1 - t + t \frac{r-1}{N}f_0;$$

we find in that event

$$E_n f_0 = \frac{N}{t} \left(\frac{N-t}{N}\right)^n + \frac{N}{t} (t-1)$$

and the expectations of the other frequencies may be similarly deduced.

Turning to the bombardment analogy, the generalisation of the Poisson schema for a single bias is almost as simple as that of the pigeon-hole schema and its formal generalisation to 2, 3, ...  $r$  biases very much easier. But, as in the pigeon-hole schema, it only proved possible (for Yule and me; more dexterous or learned mathematicians might succeed) to reach a solution *in terms of moments* for the case of a single bias.

Generalising in a different way, *i.e.* by seeking the form of the distribution when *a priori* the chances of distribution are not equal, one finds the bombardment schema leads to a very elegant solution. If we suppose the individuals (in an accident distribution) or groups to present *a priori* differences of accessibility to bombardment and that this *a priori* distribution is continuous and of skew binomial type (Pearson's Type III) the solution is eminently practicable for statistical work (see Greenwood and Yule, 1920, p. 273).

The corresponding generalisation of the pigeon-hole schema would be much less suitable. We should have to divide the  $N$  pigeon holes into groups, determine the probability that the  $n$  balls would be partitioned among the groups in a particular way by a multinomial, then work out the distribution within each group of pigeon holes receiving 0, 1, etc., balls. Only if the number of groups were very small would the operation be a practicable one.

On the whole, I think the methods set out in Greenwood and Yule's paper are fairly satisfactory. Thanks to the researches of Ethel M. Newbold, they have been improved into useful tools for the investigation of *accident* statistics. The practical handling of data relating to multiple cases of disease fulfilling the condition is more difficult because it rarely happens that the specification of the data is complete. Even when we are concerned with the statistics of trivial accidents it may be objected that our specification of the  $N_0$  frequency is incomplete or incorrect, that we may have included workpeople who were never at risk at all or have excluded others that were at risk. When we are informed that in  $N_1$  houses one case of cancer was recorded, in  $N_2$  two, and so on, we shall be in doubt as to  $N_0$ . If we take for that the whole number of houses in the administrative area less the sum of those in which cases occurred, we shall surely introduce a heterogeneity. If, on the other hand, we decide

to deduce our Poisson constant or our binomial constants from the truncated frequency we are using a method of low efficiency; in fact a layman might urge that we are playing *Hamlet* without the Prince of Denmark for the unaffected groups will usually much outnumber the sum of those affected. It is perhaps worth noting, although the point is not one of very great importance, that if we happen to have truncated data, *i.e.* data from which the  $N_0$  frequency is wanting for groups of different sizes, some light may be thrown upon the applicability of a *binomial* by the following consideration.

Suppose we were dealing with a character distributed with constant chance  $p + q = 1$ , through groups of size  $m$  and  $km$  respectively. The proportions of marked persons would naturally be identical (within the limits of error of sampling), *viz.*  $p$  in each series. But the ratios of marked persons to the number of members of groups of which at least one member of each was marked would not be identical. They would in fact be respectively  $p/(1 - q^m)$  and  $p/(1 - q^{km})$ , so that the ratio would diminish as  $k$  increased. In other words the *attack rate* would decrease as the size of the group increased. For instance, suppose that some disease were really not infectious at all but fell at random upon 10 per cent. of a population and that statistics were compiled of families of one, two and five children, all the families having at least one affected member. The attack rate in families of one would be 100 per cent., in families of two, 52.6 per cent. and in families of five, 24.4 per cent. The point is a simple one; some arguments respecting the influence of overcrowding upon mortality and morbidity have been weakened by failing to notice it<sup>1</sup>.

With these remarks I leave the kind of problem of which multiple non-fatal accidents and "cancer houses" are typical; until a more expert mathematician provides a better solution of the generalised Poisson series than Yule and I obtained, the methods discussed by us 10 years ago seem to me the most effective available.

I pass now to the other class of problem; one has groups of  $m$  individuals each and the number of cases cannot exceed  $m$  in a group. The data of observation are restricted to groups which contain at least one marked individual. This is much the most important type of problem for the epidemiologist. It has been said that the solution of every statistical problem depends on the preliminary discovery of the appropriate *Urnschema*. "Professors of probability," said Mr Keynes, "have been often and justly derided for arguing as if nature were an urn containing black and white balls in fixed proportions. Quetelet once declared in so many words: 'l'urne que nous interrogeons, c'est la nature.' But again in the history of science the methods of astrology may prove useful to the astronomer; and it may turn out to be true—reversing Quetelet's expression—that 'la nature que nous interrogeons, c'est une urne<sup>2</sup>.'" I think that this deductive method, *viz.* to imagine a way of happening, to express that mode quantitatively and to compare its consequences with the

<sup>1</sup> *Proc. Roy. Soc. Med.* 1925. Sect. of Epidem. and State Medicine, p. 38.

<sup>2</sup> Keynes, *A Treatise on Probability*, p. 428.

observed arithmetical facts, is the right course here. Current English statistical methods are perhaps *too* inductive. One sometimes spends much labour in "fitting" frequency formulae to observations having the, rather optimistic, *arrière-pensée* that in this way one can reach not merely a neat representation of the facts but also some insight into the mechanism which brought those facts about. Some years ago an English statistician took a German statistician to task because the latter had drawn some conclusions from the apparent appropriateness of Poisson's limiting binomial for the description of certain data. The critic remarked that as good, or better, fits could often be obtained by using a binomial with a fractional or negative exponent and suggested that the interpretation of such binomials needed consideration. The criticised one, in a very heated rejoinder, poured scorn upon the "formalistic" statisticians who trifled with such fantastic notions. Without seeking to meddle in that quarrel, which was a very pretty quarrel as it stood, I prefer only to use formulae here which have, or which at least I think have, a biological interpretation perfectly intelligible. To make the discussion quite clear I base it upon arithmetical examples. My friend Dr Stocks kindly provided me with Table I and we are to try to interpret the frequency distributions it contains.

Table I. *History of children aged under 10 years exposed to a case of measles in same house during 1926 epidemic in St Pancras. (Period at risk from 4 days after appearance of rash in first case, until an interval of 1 month has elapsed from onset of any case without a fresh case developing\*.)*

No. of contacts under 10 years of age (first case not included)

No. attacked during period at risk		1	2	3	4	5	6	7	8	9	10	Total
	0	340	197	84	60	25	11	3	2	1	—	723
	1	164	104	60	29	15	6	4	2	—	—	384
	2	—	57	57	25	9	4	—	3	—	1	156
	3	—	—	27	11	10	3	3	3	—	—	57
	4	—	—	—	7	1	—	2	—	—	—	10
	5	—	—	—	—	1	1	3	—	—	—	5
	6	—	—	—	—	—	1	—	—	—	—	1
	7	—	—	—	—	—	—	1	1	—	—	2
	Total	504	358	228	132	61	26	16	11	1	1	1338

\* A case developing within 3 days of first case was regarded as a simultaneous infection and not included as a contact. Contacts developing measles more than a month from onset of a preceding case were regarded as having escaped attack for purposes of this table.

Let us begin with the simplest (and, of course, certainly erroneous) hypothesis that measles is not an infectious disease at all, but that by picking out the houses in which at least one case occurred we have secured groups which were indeed exposed to some special risk. This might easily happen. A nefarious typhoid carrier might have contaminated the sealed family milk bottles of a particular group of families. Typhoid would be restricted to those families, it *might* attack at least one member of each such family and, if they were all persons who sedulously followed the spirit and letter of hygienic ritual

in eating and drinking, none of the subsequent cases would be due to intra-mural infection, the distribution must be wholly determined by personal susceptibility and dosage. In that case what sort of a distribution should we expect? Our old friend the simple binomial attracts us. Let us take for its exponent the number in house and for its  $p$  the ratio of attacked to exposed to risk. Applying this as an example to the group  $m = 3$  we have the computed distribution, which is the third column of Table II. Clearly it does not agree

Table II.

Secondary Cases	Frequency	First binomial	Second binomial	Poisson	Chain binomial
0	84	76.2	56.1	74.6	89.7
1	60	100.9	100.3	83.3	52.7
2	57	44.4	59.7	46.6	54.4
3	27	6.5	11.8	23.5	31.2

at all with observation; there are far too many "multiple" cases observed for the hypothesis. Not even if we base our binomial upon the individuals of groups of  $m = 3$  instead of upon the whole experience of all the groups do we reach a tolerable agreement. Certainly that hypothesis may be excluded. As a mere matter of empiricism the methods of the Greenwood and Yule paper were tested upon these data. The results were better than with the binomials, for instance an uncomplicated (column 5 of Table II) Poisson gave better results, but not sufficiently better for an empirical success to be able to justify an absence of biologically intelligible foundation.

Now let us go to the other extreme and imagine that *all* the cases after the first are due exclusively to personal infection. It has long been orthodox faith that the period of infectiousness is very short, let us conceptually reduce it to an instant and suppose that during an instant of time the  $m$  persons are exposed to a constant risk (furnished by the primary case), that if during this instant  $r$  are infected, then again for an instant  $m - r$  are exposed to risk. We have the conception not of a single binomial distribution but of a *chain* of binomials. Thus in the chosen illustration of  $m = 3$  we might reach a final score of three cases not uniquely as in the original use of the binomial through the term  $p^3$ , but by any one of the following:

(1) Three cases from the first exposure and of course no more; given as before by  $p^3$ .

(2) Two cases from the first exposure and then one case. The probability of the former event is  $3p^2q$ , of the latter  $p$ , because since there is but one person at risk the binomial  $(p + q)$  has unity for its exponent. Combined probability,  $3p^3q$ .

(3) One case from the first exposure and two from the second. The respective probabilities are  $3pq^2$  and  $p^2$ , the combined probability is  $3p^3q^2$ .

(4) One case from the first exposure, one from the second and one from the third; the several probabilities are  $3pq^2$ ,  $2pq$  and  $p$ , their product  $6p^3q^3$ .

Each term of the original binomial with exponent  $m$  is the first link of a



chain of binomials with diminishing exponents, except of course the first and last terms since there can be no more than  $m$  cases on the one hand, while on the other, if the initial case fails to infect any of the exposed to risk, the chain breaks at its first link.

Evidently  $r$  cases can be generated ( $r \leq m$ ) in as many ways as there are compositions of  $r$  (zero not admissible as a part), viz. in  $2^{r-1}$  ways. If  $(r_1, r_2 \dots r_s)$  be a composition, we have for the frequency of  $r_1$  primary,  $r_2$  secondary, ...  $r_s$  sth order cases,

$$\begin{aligned} & \binom{m}{r_1} p^{r_1} q^{m-r_1} \binom{m-r_1}{r_2} p^{r_2} q^{m-r_1-r_2} \dots \binom{m-r_1-r_2 \dots r_{s-1}}{r_s} \\ & \qquad \qquad \qquad p^{r_s} q^{m-r_1-r_2 \dots -r_s} q^{m-r_1-r_2 \dots r_s} \\ & = \frac{m!}{(m-r)! r_1! r_2! \dots r_s!} p^r q^{(s+1)m - [(s+1)r_1 + sr_2 + \dots + 2r_s]} \dots (1). \end{aligned}$$

When  $m$  is a small number (1) can be used directly, but my friend Dr Isserlis has devised a much more elegant way of approach.

Let us write

$$\phi_m = p^m + \binom{m}{1} p^{m-1} q \phi_1 + \binom{m}{2} p^{m-2} q^2 \phi_2 + \dots \binom{m}{s} p^{m-s} q^s \phi_s + \dots q^m \dots (2).$$

This is the required distribution. For  $m = 1$ , a single exposure exhausts the possibilities,  $\phi_1 = p + q$ , and there is one link.

When  $m = 2$ , there may be a second link on the chain:

$$\phi_2 = p^2 + 2pq\phi_1 + q^2.$$

When  $m = 3$ , we have three possible links:

$$\phi_3 = p^3 + 3p^2q\phi_1 + 3pq^2\phi_2 + q^3,$$

and so on.

Now let us expand  $\phi_m$  in powers of  $p$  by writing it  $\sum_{r=0}^{r=m} A_{m,r} p^r$ , where  $A_{m,r}$  is a function of  $q$  only.

$$(2) \equiv A_{m,m} p^m + A_{m,m-1} p^{m-1} + \dots A_{m,r} p^r + \dots A_{m,0} \dots (3).$$

In (3)  $A_{m,r}$  is the coefficient of  $p^r$ ; in (2) the coefficient of  $p^r$  is:  $\binom{m}{m-r} q^{m-r}$  multiplied by the first term of the expansion of  $\phi_{m-r}$  omitting the factor in  $p$ , +  $\binom{m}{m-r+1} q^{m-r+1}$  multiplied by the second term of the expansion of  $\phi_{m-r+1}$  omitting the factor in  $p$ , + ...  $\binom{m}{m-r+s} q^{m-r+s}$  multiplied by the  $(s+1)$ th term of the expansion of  $\phi_{m-r+s}$  omitting the factor in  $p$ ; hence in the notation of (3)

$$\begin{aligned} A_{m,r} &= \binom{m}{r} q^{m-r} A_{m-r,0} + \binom{m}{r-1} q^{m-r+1} A_{m-r+1,1} + \dots \binom{m}{1} q^{m-1} A_{m-1,r-1} \\ & \dots (4). \end{aligned}$$

$A_{m,0}$  is  $q^m$ , so (4) enables us to calculate the  $A$ 's step by step. Thus:

$$A_{m,1} = \binom{m}{1} q^{m-1} A_{m-1,0} = m q^{m-1} q^{m-1} = m q^{2(m-1)};$$

$$\begin{aligned} A_{m,2} &= \binom{m}{2} q^{m-2} A_{m-2,0} + \binom{m}{1} q^{m-1} A_{m-1,0} \\ &= \binom{m}{2} q^{m-2} q^{m-2} + m q^{m-1} (m-1) q^{2(m-2)} \\ &= \binom{m}{2} q^{2(m-2)} (1 + 2q^{m-1}). \end{aligned}$$

The values up to  $A_{m,6}$  are:

$$A_{m,0} = q^m;$$

$$A_{m,1} = \binom{m}{1} q^{2(m-1)};$$

$$A_{m,2} = \binom{m}{2} q^{2(m-2)} (1 + 2q^{m-1});$$

$$A_{m,3} = \binom{m}{3} q^{2(m-3)} (1 + 3q^{m-2} + 3q^{m-1} + 6q^{2m-3});$$

$$A_{m,4} = \binom{m}{4} q^{2(m-4)} (1 + 4q^{m-3} + 6q^{m-2} + 4q^{m-1} + 12q^{2m-5} + 12q^{2m-4} + 12q^{2m-3} + 24q^{3m-6});$$

$$\begin{aligned} A_{m,5} &= \binom{m}{5} q^{2(m-5)} (1 + 5q^{m-4} + 10q^{m-3} + 10q^{m-2} + 5q^{m-1} + 20q^{2m-7} \\ &\quad + 30q^{2m-6} + 50q^{2m-5} + 30q^{2m-4} + 20q^{2m-3} \\ &\quad + 60q^{3m-9} + 60q^{3m-8} + 60q^{3m-7} + 60q^{3m-6} \\ &\quad + 120q^{4m-10}); \end{aligned}$$

$$\begin{aligned} A_{m,6} &= \binom{m}{6} q^{2(m-6)} (1 + 6q^{m-5} + 15q^{m-4} + 20q^{m-3} + 15q^{m-2} + 6q^{m-1} \\ &\quad + 30q^{2m-9} + 60q^{2m-8} + 120q^{2m-7} + 120q^{2m-6} \\ &\quad + 120q^{2m-5} + 60q^{2m-4} + 30q^{2m-3} + 120q^{3m-12} \\ &\quad + 180q^{3m-11} + 300q^{3m-10} + 360q^{3m-9} \\ &\quad + 300q^{3m-8} + 180q^{3m-7} + 120q^{3m-6} + 360q^{4m-14} \\ &\quad + 360q^{4m-13} + 360q^{4m-12} + 360q^{4m-11} \\ &\quad + 360q^{4m-10} + 720q^{5m-15}). \end{aligned}$$

The fractional frequency, or probability, that, after a complete exposure, out of  $m$ ,  $r$  will be infected,  ${}_m f_r$  say, is merely  $A_{m,r}$  multiplied by  $p^r$ .

Hence the respective  $f$ 's can be computed. If  $m = r$ , (4) becomes

$$A_{m,m} = 1 + \binom{m}{1} q A_{1,1} + \binom{m}{2} q^2 A_{2,2} + \dots + \binom{m}{m-1} q^{m-1} A_{m-1,m-1},$$

and we can compute  $A_{1,1}$ ,  $A_{2,2}$ , etc., checking the previous calculation.

In actual practice, unless a table of  $f$ 's for different values of  $m$  and  $p$  has

been computed, the mean number of cases will be needed; it can be calculated without using  $A_{mm}$ . We see from (3) that the mean, which we may denote by  $\phi_m$ , is

$$A_{m,1}p + 2A_{m,2}p^2 + \dots,$$

$$\text{i.e.} \quad p\phi'_m \text{ if } \phi'_m = \frac{d\phi_m}{dp};$$

from (2) we have

$$\begin{aligned} \phi'_m &= mp^{m-1} + (m-1) \binom{m}{1} p^{m-2} q \phi_1 + \dots \\ &+ \binom{m}{1} p^{m-1} q \phi'_1 + \binom{m}{2} p^{m-2} q^2 \phi'_2 + \dots \end{aligned}$$

Since  $\phi_s = 1$  for all values of  $s$ , the first line =  $m$ . Hence

$$\bar{\phi}_m = \binom{m}{1} p q^{m-1} \bar{\phi}_{m-1} + \binom{m}{2} p^2 q^{m-2} \bar{\phi}_{m-2} + \dots \binom{m}{1} p^{m-1} q \bar{\phi} + mp \quad \dots (2a).$$

Now if we write

$$\bar{\phi}_m = B_{m,m} p^m + B_{m,m-1} p^{m-1} + \dots B_{m,1} p \quad \dots (3a),$$

and equate coefficients as before between (2a) and (3a), we find that

$$B_{m,r} = \binom{m}{r} q^{m-r} B_{m-r,0} + \binom{m}{r-1} q^{m-r+1} B_{m-r+1,1} + \dots \binom{m}{1} q^{m-1} B_{m-1,r-1} \quad \dots (5)$$

of the same form as (4). In (4)  $A_{m,0} = q^m$ , in (5)  $B_{m,1} = m$ , giving

$$B_{m,2} = \binom{m}{1} q^{m-1} B_{m-1,1} = \binom{m}{1} q^{m-1} (m-1), \text{ etc.}$$

So

$$\begin{aligned} \frac{B_{m,1}}{A_{m,0}} &= \frac{m}{q^m}, \quad \frac{B_{m,2}}{A_{m,1}} = \frac{m-1}{q^{m-1}} \dots; \\ \frac{B_{m,r}}{A_{m,r-1}} &= \frac{m-r+1}{q^{m-r+1}}. \end{aligned}$$

Hence

$$\begin{aligned} \bar{\phi}_m &= p B_{m,1} + p^2 B_{m,2} + \dots \\ &= p \frac{m}{q^m} A_{m,0} + p^2 \frac{m-1}{q^{m-1}} A_{m,1}, \text{ etc.} \end{aligned}$$

These formulae enable us to solve the problem with semi-mechanical effort in any case. Formulae expressed in terms of  $p$  and  $q$  are not, however, suitable for use. In order to obtain a suitable value of  $q$  from the statistics, we require to express the mean of the statistics as a function of the  $p$  and  $q$  and, that its accuracy may be readily checked, it is better to reduce to terms of  $q$  (or  $p$ ) only. Evidently if the reduction is correct, the sum of the coefficients of powers of  $q$  and the constant term must vanish, for if  $q = 1$ , the mean must be zero. Similarly, the term free of  $q$  must be equal to  $m$ , for if  $p = 1$ , the mean must be  $m$ . The values of the frequencies and the means for values of  $m$  from 2 to 5 are given below.

$$m = 2.$$

$$f_0 = q^2.$$

$$f_1 = 2q^2(1 - q).$$

$$f_2 = 1 - 3q^2 + 2q^3.$$

$$\text{Mean} = 2 - 4q^2 + 2q^3.$$

$$m = 3.$$

$$f_0 = q^3.$$

$$f_1 = 3q^4(1 - q).$$

$$f_2 = 3q^2(1 - 2q + 3q^2 - 4q^3 + 2q^4).$$

$$f_3 = 1 - 3q^2 + 5q^3 - 12q^4 + 15q^5 - 6q^6.$$

$$\text{Mean} = 3 - 3q^2 + 3q^3 - 15q^4 + 18q^5 - 6q^6.$$

$$m = 4.$$

$$f_0 = q^4.$$

$$f_1 = 4q^6(1 - q).$$

$$f_2 = 6q^4(1 - 2q + q^2 + 2q^3 - 4q^4 + 2q^5).$$

$$f_3 = 4q^2(1 - 3q + 6q^2 - 7q^3 + 12q^5 - 21q^6 + 18q^7 - 6q^8).$$

$$f_4 = 1 - 4q^2 + 12q^3 - 31q^4 + 40q^5 - 10q^6 - 56q^7 + 108q^8 - 84q^9 + 24q^{10}.$$

$$\text{Mean} = 4 - 4q^2 + 12q^3 - 40q^4 + 52q^5 - 24q^6 - 60q^7 + 132q^8 - 96q^9 + 24q^{10}.$$

$$m = 5.$$

$$f_0 = q^5.$$

$$f_1 = 5q^8(1 - q).$$

$$f_2 = 10q^6(1 - 2q + q^2 + 2q^4 - 4q^5 + 2q^6).$$

$$f_3 = 10q^4(1 - 3q + 3q^2 + 2q^3 - 6q^4 + 6q^6 + 3q^7 - 18q^8 + 18q^9 - 6q^{10}).$$

$$f_4 = 5q^2(1 - 4q + 10q^2 - 14q^3 + 5q^4 + 16q^5 - 32q^6 + 26q^7 - 20q^8 + 60q^9 - 132q^{10} + 156q^{11} - 96q^{12} + 24q^{13}).$$

$$f_5 = 1 - 5q^2 + 20q^3 - 60q^4 + 99q^5 - 65q^6 - 80q^7 + 205q^8 - 125q^9 + 20q^{10} - 290q^{11} + 820q^{12} - 960q^{13} + 540q^{14} - 120q^{15}.$$

$$\text{Mean} = 5 - 5q^2 + 20q^3 - 70q^4 + 125q^5 - 115q^6 - 60q^7 + 230q^8 - 110q^9 - 80q^{10} - 240q^{11} + 960q^{12} - 1140q^{13} + 600q^{14} - 120q^{15}.$$

Table III. *Frequencies of secondary cases of measles, compared with Chain Binomials (bracketed figures).*

	$m=2$		$m=3$	
0	197 (198.3)	120 (122.4)	84 (89.7)	37 (40.8)
1	104 (101.4)	93 (88.2)	60 (52.7)	34 (27.9)
2	57 (58.3) $P=0.75$	86 (88.4) $P=0.58$	57 (54.4)	42 (42.7)
3	—	—	27 (31.2) $P=0.36$	36 (37.6) $P=0.42$
	$m=4$		$m=5$	
0	60 (59.54)	—	25 (26.86)	—
1	29 (28.87)	—	15 (12.42)	—
2	25 (24.45)	—	9 (10.63)	—
3	11 (14.35)	—	10 (6.90)	—
4	7 (4.79) $P=0.61$	—	1 (3.30)	—
5	—	—	1 (0.89) $P=0.42$	—

The sixth column of Table II contains the values deduced on this hypothesis; they plainly agree much better with the observations than the binomial terms.

Table IV.

No. of children under 10 years of age, not having had measles, exposed to infection from a case in same house during 1926 epidemic (St Pancras)

No. attacked (at least 4 days after first case and within a month of last case in house)	1	2	3	4	5	6	7	Total
	0	322	120	37	12	3	1	495
	1	238	93	34	10	4	—	379
	2	—	86	42	5	5	2	141
	3	—	—	36	9	9	2	56
	4	—	—	—	7	2	1	10
	5	—	—	—	—	1	1	2
	6	—	—	—	—	—	1	1
	7	—	—	—	—	—	2	2
	Total	560	299	149	43	24	8	1089
Total children in above houses	560	598	447	172	120	48	42	1987
No. of these attacked	238	265	226	75	54	25	32	915

In Table III the comparison is extended to all values of  $m$  up to  $m = 5$  and to two other sets of data, for the cases of  $m = 2$  and  $m = 3$  derived from Table IV (also supplied by Dr Stocks) which relates to exposed to risk somewhat more stringently selected.

Not one of the six values of  $P$  (deduced by entering Elderton's table for  $n' =$  the number of frequencies less one, since *two* degrees of freedom have been absorbed) is less than 0.35, so that the agreement between fact and fancy is statistically respectable. One might anticipate some improvement by dropping the condition that  $p$  is constant throughout the chain, *i.e.* the condition that the danger of a secondary case to the exposed to risk is as great as that of a primary. This condition seems biologically improbable, for we should, especially in view of Dr Stocks' recent work, expect the factor of latent immunisation to play some part. But unless  $m$  were larger than it is likely to be in practice, it would be futile to seek to deduce a series of  $p$ 's one for each remove from the first link. The deduction of each new  $p$  would mean the absorption of one more degree of freedom, and we should tend to reach a meaningless, because compulsory, concordance between theory and observation.

I think, speaking as a biological statistician, that this arithmetical device is as successful in describing the facts of measles transmission as we can reasonably expect, and that its application to similar data of scarlet fever, smallpox, diphtheria and whooping-cough would be illuminating. In scarlet fever, one would expect a similar result but with  $p$  smaller. Perhaps the ratios of the deduced  $p$ 's might be an acceptable measure of the infectiousness of scarlet fever in terms of the standard of measles.

In diphtheria the position is more complex, since the period of infectious-

ness of each case (assuming, of course, that the patients are not removed from contact with the exposed to risk) is *not* limited to a short period of time. Under those circumstances the conception of a series of links in a chain is inappropriate. We should, I think, need to return to the conception of a continuous bombardment. As, however, I have no data, speculation is idle. I confine myself to the present, very modest, contribution to the study of infectiousness in diseases appertaining roughly to the measles class.

The arithmetical computation from the formulae given is not difficult. The longest stage is the approximation to  $q$ , by the use, for instance, of Newton's approximation. Actually putting  $q^m = f_0/F$ , where  $f_0$  is the frequency of groups with no secondaries and  $F$  the total number of groups, gives a first approximation close enough for one or two applications of the Newtonian approximation to suffice.

Taking as an illustration the most difficult case, we have:

Cases	Frequency
0	25 (26.86)
1	15 (12.42)
2	9 (10.62)
3	10 (6.90)
4	1 (3.30)
5	1 (0.89)
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The mean is 1.1803279, so that the equation for  $q$  is

$$120q^{15} - 600q^{14} + 1140q^{13} - 960q^{12} + 240q^{11} + 80q^{10} + 110q^9 - 230q^8 + 60q^7 \\ + 115q^6 - 125q^5 + 70q^4 - 20q^3 + 5q^2 - 3.8196721 = 0.$$

$25/61 = 0.409836$  must be approximately equal to  $q^5$  and  $0.836608$  is approximately equal to  $\sqrt[5]{0.409836}$ . Hence  $0.84$  may be taken as a first approximation to the value of  $q$ .

Substituting in the equation and in its first differential we have

$$f(x) = -0.0877237,$$

$$f'(x) = 10.102029,$$

giving

$$f(x)/f'(x) = -0.0086838.$$

A second approximation is  $0.84868$ , and proceeding as before we reach  $0.84873$  as a sufficiently near approximation for our purpose. Substituting this value of  $q$  in the formulae, one reaches the values given in parentheses.

For purposes of *rough* estimation a table of values such as Table V will be found useful. The intervals, however, are too large for it to be possible by interpolation to reach accurate results. Thus, taking the example just worked out, interpolating for the values of the mean by taking  $0.5086$  of the terms of the series for  $q = 0.9$  and  $0.4914$  times the values corresponding in the series for  $q = 0.8$ , one reaches  $28.1$ ,  $11.7$ ,  $9.5$ ,  $6.6$ ,  $3.8$  and  $1.3$  which are not close approximations to the correct values although, perhaps, sufficient for rough estimations. Since, as already stated, I have no other data than those

provided by Dr Stocks, which reach a respectable standard of accuracy, I cannot myself test the value of the method on the findings in other diseases. But a single, only half-serious experiment, is worth mentioning. Table VI is taken

Table V.

$q =$	...	.1	.2	.3	.4	.5	.6	.7	.8	.9
$m = 2 \left\{ \begin{array}{l} f_0 \\ f_1 \\ f_2 \end{array} \right.$	$f_0$	.01	.04	.09	.16	.25	.36	.49	.64	.81
	$f_1$	.018	.064	.126	.192	.25	.288	.294	.256	.162
	$f_2$	.972	.896	.784	.648	.50	.352	.216	.104	.028
	Mean	1.962	1.856	1.694	1.488	1.25	.992	.726	.464	.218
$m = 3 \left\{ \begin{array}{l} f_0 \\ f_1 \\ f_2 \\ f_3 \end{array} \right.$	$f_0$	.001	.008	.027	.064	.125	.216	.343	.512	.729
	$f_1$	.0003	.0038	.0170	.0461	.0938	.1555	.2161	.2458	.1968
	$f_2$	.0248	.0829	.1561	.2281	.2813	.2972	.2620	.1751	.0637
	$f_3$	.9739	.9052	.7999	.6618	.5000	.3313	.1790	.0671	.0105
	Mean	2.9717	2.8854	2.7289	2.4877	2.1563	1.7437	1.2769	.7974	.3557
$m = 4 \left\{ \begin{array}{l} f_0 \\ f_1 \\ f_2 \\ f_3 \\ f_4 \end{array} \right.$	$f_0$	.0001	.0016	.0081	.0256	.0625	.1296	.2401	.4096	.6561
	$f_1$	.0000	.0002	.0020	.0098	.0313	.0746	.1412	.2097	.2126
	$f_2$	.0005	.0062	.0251	.0624	.1172	.1782	.2186	.1990	.0968
	$f_3$	.0301	.0939	.1686	.2396	.2891	.2944	.2385	.1315	.0297
	$f_4$	.9693	.8981	.7961	.6626	.5000	.3232	.1616	.0502	.0049
	Mean	3.9685	3.8866	3.7427	3.5037	3.1328	2.6069	1.9403	1.2030	.5147
$m = 5 \left\{ \begin{array}{l} f_0 \\ f_1 \\ f_2 \\ f_3 \\ f_4 \\ f_5 \end{array} \right.$	$f_0$	.0000	.0003	.0024	.0102	.0313	.0778	.1681	.3277	.5905
	$f_1$	.0000	.0000	.0002	.0020	.0098	.0336	.0865	.1678	.2152
	$f_2$	.0000	.0004	.0036	.0155	.0439	.0940	.1567	.1908	.1229
	$f_3$	.0007	.0084	.0307	.0707	.1257	.1829	.2103	.1646	.0527
	$f_4$	.0343	.0999	.1724	.2410	.2893	.2915	.2255	.1080	.0161
	$f_5$	.9649	.8910	.7906	.6606	.5000	.3203	.1530	.0412	.0026
	Mean	4.9641	4.8804	4.7422	4.5120	4.1321	3.5376	2.6975	1.6811	.6966

Table VI.

Households having cases	...	1	2	3	4	5	6	7	Total
No. of households	...	234	101	57	30	12	4	2	440
No. of persons	...	1083	513	326	186	78	32	19	2237
Average no. of persons per house		4.63	5.01	5.72	6.2	6.5	8	9.5	---

from the *Ministry of Health's Report on the Pandemic of Influenza, 1918-19*. I arbitrarily simplified the table and assumed that it referred to groups of five in family and would thus read:

Secondary cases	No. of families
0	234
1	101
2	57
3	30
4	12
5	6
	<hr/> 440

The mean is 0.87. Treated as a binomial with exponent 5 we should have  $p = 0.174$  and  $q = 0.826$ . This would yield as frequencies 169.2, 178.2, 75.1, 15.8, 1.7 and 0.1. Clearly preposterous values. The Poisson frequencies are 184.5, 160.2, 69.7, 20.3, 4.4 and 0.9 (grouping 5 and onwards), almost equally preposterous. Interpolating for a "chain" we have 239.4, 91.0, 59.3, 31.9, 14.2, 4.1; a quite reasonable fit and, as we have seen, this is not the best fit which we could obtain (the experiment is too trivial to justify the labour of

careful fitting). One may say that the influenza of 1918-19 seems to have behaved rather like measles in its domestic evolution. However, the object of this paper is to bring to the notice of others a method, not to discuss, without data, epidemiological results.

To Dr Isserlis I owe not only the elegant demonstration quoted but helpful criticism of the whole idea and Mr W. J. Martin has been indefatigable in sparing me laborious arithmetic. To our valued colleague the late Mr H. E. Soper, I owe the painless destruction of various other weakly dream children brought to birth during this study and the encouragement that this brat might be worth preservation.

## REFERENCES.

- GREENWOOD, M. and YULE, G. U. (1920). *J. Roy. Stat. Soc.* **83**, 255-79.  
MAYNARD, G. D. and TROUP, J. McD. (1911). *Biometrika*, **8**, 396-404.  
NEWBOLD, E. M. (1927). *J. Roy. Stat. Soc.* **90**, 487-535.  
PEARSON, K. (1911). *Biometrika*, **8**, 405-12.  
— (1913). *Ibid.* **9**, 28-33.

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## OBSERVATIONS ON THE WEIL-FELIX REACTION IN TSUTSUGAMUSHI DISEASE<sup>1</sup>.

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(With 3 Charts.)

### INTRODUCTION.

CASES of a disease of the typhus group, with symptoms very similar to those of the tsutsugamushi disease in Japan, have been observed in certain areas of the east coast of Sumatra ever since Schüffner in 1909 published his first observations on this disease (then called pseudo-typhoid). Although not exactly alike in epidemiology and clinical features (see Schüffner, 1909; Maasland, 1926; Kawamura, 1930), it is generally admitted that these two mite-borne diseases belong to the same group.

In 1925 Fletcher and Lesslar published several observations on another typhus-like fever, "tropical typhus," the clinical symptoms of which were much like those of tsutsugamushi, type Schüffner, but differed mainly in two ways; firstly as to the primary ulcer and hubo, which were always absent in their tropical typhus cases, while secondly there was no evidence that a mite was the vector as is the case with the tsutsugamushi disease. Fletcher and his co-workers stated further that two types of tropical typhus occurred with slight variation in the course of the fever, the main difference being the Weil-Felix reaction. In the urban type, "shop" typhus (Fletcher, 1930), agglutinins were formed in the patient's serum against the ordinary *Proteus* X 19 strain, type "Warsaw." In the rural type, "scrub" typhus, a positive agglutination reaction occurred with the non-indologenic variety called the "Kingsbury" strain. In all these latter cases the agglutination of the patient's serum with the ordinary *B. proteus* X 19 strain invariably gave a negative result.

The observations of Fletcher and his co-workers were soon confirmed in other localities. In Sumatra I was able to observe several cases of tropical typhus of both types (1929). Since cases of scrub typhus occurred side by side with cases of tsutsugamushi, it was of importance to see whether a positive Weil-Felix reaction would occur during the course of tsutsugamushi disease and whether or not it would assist in the differential diagnosis of the two diseases.

<sup>1</sup> A short note on these observations will be published in the *Geneeskundig Tijdschrift voor Nederlandsch Indië*.

## REVIEW OF EARLIER LITERATURE.

In cases of tsutsugamushi disease in Japan, the Weil-Felix reaction is stated to be negative or of so low a titre as to have no diagnostic value (Ishiwara and Ogata, 1923; Kawamura, 1926, 1930). Apparently these authors have used the customary indologenic strain of X 19. In a recent publication, Fletcher, Lesslar and Lewthwaite (1929), discussing the differential diagnosis between scrub typhus and tsutsugamushi disease in the Federated Malay States, state that, "...the agglutination reaction with the non-indologenic strain of *B. proteus*, known as the Kingsbury strain, also serves to separate scrub-typhus from the tsutsugamushi disease, because the titre of agglutination is far higher in the former than in the latter; at the same time this test shows the close relationship of the two fevers, because tsutsugamushi is the only disease, except scrub-typhus, in which the blood has been found to agglutinate the Kingsbury strain in dilutions higher than 1 in 200...." The highest titre Fletcher found in three out of seven cases was stated to be 1 in 240.

## THE AUTHOR'S OBSERVATIONS.

The present study deals with observations on forty-five cases of tsutsugamushi, type Schüffner. Since 1926, agglutinations with suspensions of the "Warsaw" and "Kingsbury" strains have been performed with most of the samples of human sera sent in, for diagnostic purposes (Widal tests, etc.), to the Pathologisch Laboratorium in Medan. The sera were sent in from different cases of fever, amongst which there have been every year several well-established cases of tsutsugamushi disease. However, since nearly all these sera were sent in during the first days of the disease, those agglutinations invariably have had a negative result. And as soon as the primary ulcer was found, for diagnostic purposes it was not necessary to send in serum for agglutination tests during a later phase of the disease.

In order to investigate this matter further, a series of cases of tsutsugamushi disease have been examined as far as possible every few days. All these cases were diagnosed and treated by Dr M. Straub of the Bangkattan Hospital of the Deli Maatschappij. I am very much indebted to him for sending me sera of his patients on several occasions, and for allowing me to make use of his case records.

From the beginning of 1929 up to March 1930 sera from fifty tsutsugamushi cases have been received. In this communication I have not made use of the notes of five cases, since in four of these it was thought afterwards that the occurrence of a primary ulcer had not been established with certainty. The fifth case had a primary ulcer, but showed, after a series of totally negative agglutination tests, a positive agglutination on the fifteenth day of the disease with the "Warsaw" strain up to 1 in 1000, while on the seventeenth and nineteenth days again a negative agglutination was noted. As all the other positive

agglutinations have occurred with the "Kingsbury" strain, this result may probably have been misrecorded.

The other forty-five cases all showed a typical primary ulcer and nearly all of them a corresponding bubo near the site of the ulcer. The number of agglutination tests performed in these cases is set out in Table I.

Table I.

No. of tests performed in each case	Cases of <i>tsutsugamushi</i> where the Weil-Felix test with the "Kingsbury" strain	
	Became positive	Remained negative
1	2	2
2	3	1
3	3	4
4	9	2
5	6	4
6	3	1
7	1	.
8	3	.
11	.	1
	<hr/> 30	<hr/> 15

It will be seen that only in thirty out of forty-five cases was the agglutination positive with the "Kingsbury" strain during some phase of the disease. In fifteen cases the agglutination was negative. It might naturally be presumed, that in those cases, where only one or two tests were performed during the course of the disease, an agglutination made during a later phase might have been positive. However, some observations, which are discussed later on, show very clearly that in some cases no agglutinins could be detected during the whole course of the disease.

The following technique of agglutination tests has been employed. Saline suspensions of the motile flagellated "Warsaw" and "Kingsbury" strains were used after growth for 24 hours on sloped agar. The bacillary suspensions were killed by 1 per cent. formalin. One-half of each serum sample was heated for an hour at 56° C. and with the heated and unheated serum samples agglutination tests were performed in dilutions up to 1 in 1000. The object of heating a part of the serum was to see whether there was any difference in titre between the unheated and the heated serum portion. According to Prausnitz (1920), the specific agglutinins in the serum of typhus patients are for the greater part destroyed by heating the serum, while the *proteus*-agglutinins, due to infection with some ordinary *B. proteus vulgaris*, are heat stable<sup>1</sup>. This difference is due to the different resistance to heat of O and H agglutinins (Weil and Felix, 1917; Felix and Olitzki, 1929). For their differentiation in routine diagnosis it is recommended by Felix (1930) to use side by side suspensions of O and H variants of *B. proteus* X.

<sup>1</sup> In my previously quoted article on tropical typhus, some agglutination records show a very marked difference in titre between the unheated and heated serum tests.

*cases of tsutsugamushi disease.*

Year	No.	Day of disease	Serum		Year	No.	Day of disease	Serum	
			Un-heated	Heated				Un-heated	Heated
1929	675	6	100	.	1930	702	6	—	.
		13	100	.			11	25	.
		18	—	.			13	50	.
	891	12	100	.			16	50	.
		17	100	.			18	25	.
	1,872	7	—	.			20	25	.
		12	—	.			22	25	.
		19	1000	250			28	—	.
	1,923	8	1000	1000		905	6	—	.
		8	—	.			10	50	250
	2,351	15	—	—			12	500	250
		20	1000	500			14	500	100
		25	100	50			16	250	100
		25	100	50			18	100	50
	2,396	16	250	50		1,061	7	—	.
		14	1000	1000			11	—	.
	4,236	23	1000	500			13	50	—
		31	25	—			15	50	25
		31	25	—			17	25	—
	5,769	5	100	50		1,179	12	—	—
		7	250	250			14	500	100
		11	250	100		1,382	11	50	25
		13	250	100			13	250	100
		15	100	25		1,644	15	250	.
		17	50	25			17	1000	.
	6,395	5	—	.			19	500	.
		13	250	.			21	500	.
		15	500	.		1,741	15	1000	250
		18	50	.			18	1000	500
		20	—	.			20	1000	1000
	9,509	6	—	—		2,201	27	100	50
		19	100	50			8	—	—
		21	—	—			18	500	50
		23	—	—		2,248	20	250	—
		25	—	—			22	50	—
	13,244	4	—	.			26	25	25
		11	—	—		2,249	10	—	—
		16	100	50			16	1000	1000
		18	1000	100			17	1000	1000
		20	1000	250			19	1000	250
		22	1000	100		2,470	6	—	—
		24	1000	100			11	25	—
		26	500	50			14	1000	500
	14,120	11	1000	1000			16	—	—
		13	1000	500			18	—	—
		15	1000	500		2,499	4	—	—
		17	500	50			9	—	—
1930	640	6	100	.			11	250	100
		8	1000	.			13	—	—
		10	1000	.			7	—	—
		12	1000	.		2,507	9	25	—
	641	4	—	—			11	100	—
		6	—	—			13	500	250
		8	—	—			15	250	25
		10	—	—			17	100	—
		12	500	100		2,600	19	—	—
		14	1000	500			9	25	—
		16	1000	1000			11	100	—
		18	1000	1000			13	250	—
	732	6	—	—			17	250	—
		7	—	—		3,789	5	—	—
		9	50	—			12	100	.
		11	500	.			14	250	100
		13	1000	.			16	50	25
		15	1000	.			18	50	—

\* Alcoholic suspension.

The titre recorded is the highest dilution in which the agglutination was visible to the naked eye. Except in the last two cases, where alcoholic suspensions also were used, all agglutinations were performed with formalin suspensions. Dilutions higher than 1 in 1000 were not tested. — signifies negative. . signifies not done.

## CASES WITH POSITIVE AGGLUTINATION TESTS.

Table II shows the agglutination results of all those cases where a positive agglutination occurred during the course of the disease. The agglutination tests with both the heated and unheated sera and the "Warsaw" strain, which were invariably negative, are not recorded in this table. In the last two cases of Table II agglutinations were also performed with the unheated serum and alcoholic suspensions of the two strains, following the technique of Bien and Sonntag (1917). It will be seen that with these alcoholic (O) suspensions, the titre was slightly lower than that reached when formalin suspensions were used.

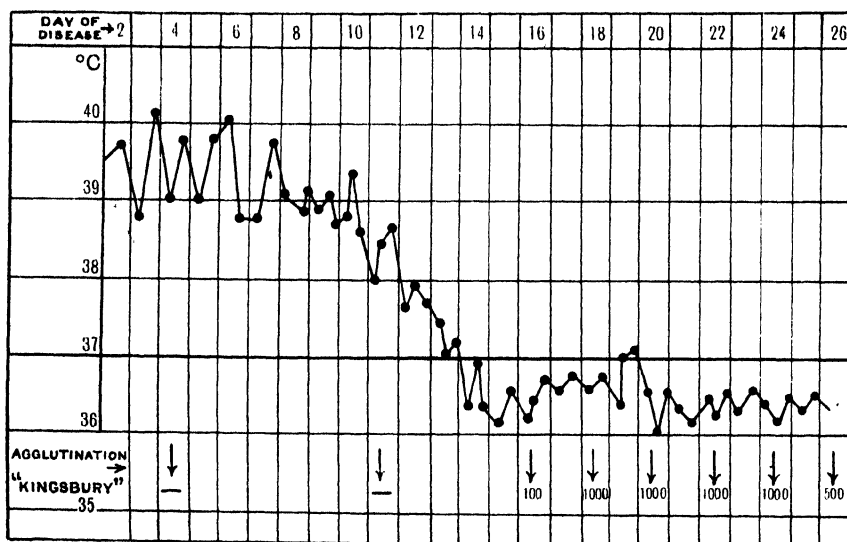


Chart I. No. 13,244. Javanese labourer,  $\pm$  30 years. In hospital Dec. 2nd-26th, 1929. On admission a slight patchy rash is noticed, spread over face, breast and back. In the right fossa inguinalis a tsutsugamushi ulcer is present, also a painful swollen bubo. Heart and lungs normal, spleen swollen. Blood count. 5700 leucocytes. Dec. 4th. Widal: typhoid 1/50. Cultures of blood, faeces and urine negative for *B. typhosus*. Urine trace albumen, diazo positive, urobiline positive. Dec. 7th. Rash faintly visible. Dec. 11th. Ulcer healed. Widal: typhoid 1/25. Blood, faeces and urine culture negative for *B. typhosus*. For Weil-Felix reaction see chart.

It will be seen from Table II that, in some cases, only one or two agglutination tests could be performed. In these cases it is difficult to prove that the recorded positive reaction is due to the tsutsugamushi infection and is not the result of an earlier infection with the virus of tropical typhus. But in twenty-four cases there is a definite rise in the titre during the course of the disease. Chart I, accompanied by short notes of case No. 13,244, serves as an example.

During the course of the fever no agglutinins had been formed on the fourth nor on the eleventh day of illness. On the sixteenth day an agglutination titre of 1 in 100 was noted in the unheated serum, which rose to 1 in 1000 on the eighteenth day and persisted till the twenty-fifth day. It might even have

been higher during that interval, but unfortunately higher dilutions were not used. The last test in this case was made on the day of the patient's dismissal, when the titre was found to have dropped to 1 in 500.

Another interesting case was No. 641. (See Chart II.)

This patient was first admitted with a fever during the course of which a well-marked positive Weil-Felix reaction with the "Kingsbury" strain developed. Since the titre of agglutination showed a rise on two following occasions, while the most careful inspection revealed no trace of an ulcer or

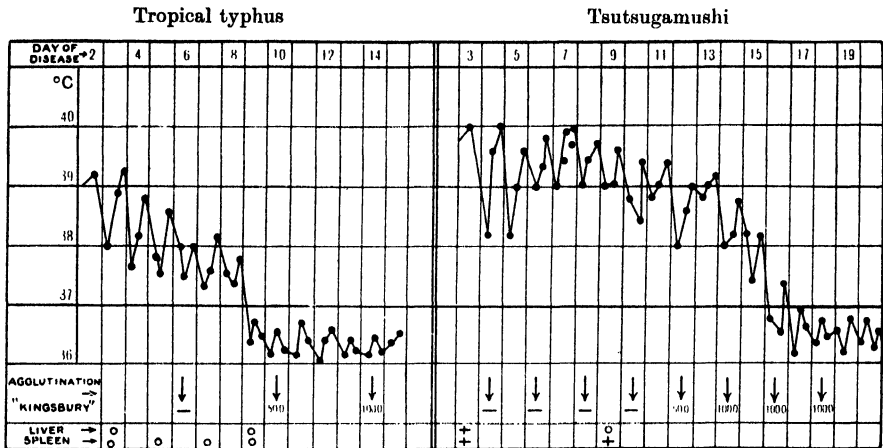


Chart II. No. 641. Javanese labourer,  $\pm$  24 years. In hospital June 7th-29th, 1929, and Dec. 30th, 1929-Jan. 21st, 1930. On admission patient complains of fever, headache. Heart normal, slight bronchitis. In urine no albumen, diazo, and trace urobiline. In blood malaria negative. 10,800 leucocytes. Cultures of blood, faeces and urine negative for *B. typhosus*. Widal on June 11th, typhoid 1/50, para-A 1/250 (patient had been vaccinated with T.A.B. in April, 1929). For course of fever and Weil-Felix reaction see chart. Six months later the same man is re-admitted with high fever. On the right side of the scrotum a typical ulcer with bubo. Spleen swollen, heart and lungs normal. Widal twice negative. In urine trace albumen, diazo and urobiline reaction both positive. On Jan. 8th a parotitis developed which healed in a few days. See chart and text for course of fever and Weil-Felix reaction.

bubo, the diagnosis tropical typhus (type "Kingsbury") was made. Six months later, the same man was readmitted to the hospital with high fever, showing a typical tsutsugamushi ulcer on the scrotum. Both times the man had worked in an area of newly cut secondary growth, where more cases of tsutsugamushi and tropical typhus occurred side by side. Till the twelfth day of his illness, the serum of this patient showed no trace of agglutinins for the "Kingsbury" strain; they had disappeared during the interval of six months. Thereafter, a strong agglutination up to 1 in 1000 was noted, still present at the time of his dismissal<sup>1</sup>.

<sup>1</sup> As far as I know, this is the first evidence of the occurrence of these two diseases of the typhus group in the same individual after an interval as short as six months. In this case, the infection with the first virus (whether tropical typhus or tsutsugamushi without a primary ulcer) gave no lasting immunity against a later infection with the tsutsugamushi virus. Similar observations have been published from Japan (Kawamura, 1930).

## CASES WITH NEGATIVE AGGLUTINATION TESTS.

As stated previously (see Table I), in fifteen out of forty-five patients suffering from tsutsugamushi disease the serum yielded negative reactions with the "Kingsbury" strain. In some of these cases the test has not been applied often enough to allow any definite conclusion to be drawn from the negative results recorded. But in other cases agglutinations were performed at a stage of the disease when a positive agglutination might have been expected, judging from those cases where the formation of agglutinins did take place. For comparison all positive and negative results, according to the day of the disease, have been recorded in Table III, while Chart III relates to a case with a negative Weil-Felix reaction throughout the course of the disease.

Table III. *Agglutination tests with the "Kingsbury" strain according to day of disease.*

Day of disease	Titre of agglutination							Total positive
	Negative	1/25	1/50	1/100	1/250	1/500	1/1000	
3	1	.	.	.	.	.	.	.
4	3	.	.	.	.	.	.	.
5	7	.	.	1	.	.	.	1
6	9	.	.	2	.	.	.	2
7	8	.	.	.	1	.	.	1
8	4	.	.	.	.	.	2	2
9	2	2	1	.	.	.	.	3
10	6	.	1	.	.	.	1	2
11	9	2	1	2	2	1	1	9
12	3	.	.	2	1	2	1	6
13	6	.	2	1	4	1	2	10
14	5	.	.	.	1	2	3	6
15	4	.	1	1	2	1	3	8
16	4	.	2	1	2	.	2	7
17	4	1	1	2	1	1	2	8
18	7	1	2	1	.	1	3	8
19	6	.	.	1	.	1	2	4
20	5	1	.	.	1	.	3	5
21	2	.	.	.	.	2	.	2
22	2	1	1	.	.	.	.	2
23	6	.	.	.	.	.	2	2
24	3	.	.	.	.	.	1	1
25	1	.	.	1	.	.	.	1
26	.	1	.	.	.	1	.	1
27	.	1	.	1	.	.	.	2
28	2	.	.	.	.	.	.	.
29	.	.	.	.	.	.	.	.
30	.	.	.	.	.	.	.	.
31	.	1	.	.	.	.	.	1
<hr/>								
	109	11	12	16	15	13	28	94

It will be seen that this case was clinically a typical tsutsugamushi. Instances of failure to form agglutinins have been recorded in classical typhus fever. There the disease either ran an extremely mild course or the case was a very toxic one, nearly invariably ending fatally, where we may presume, that the body failed to react with the formation of antibodies (for references see Felix, 1930). In our cases the reason for failure of production of antibodies is not known. All that can be said is that these cases with negative agglutination reactions were neither extremely mild nor very toxic, the mortality being nil;

the fever in two of these cases lasted for 22 and 26 days respectively, while in the others the duration of the fever was between 7 and 15 days.

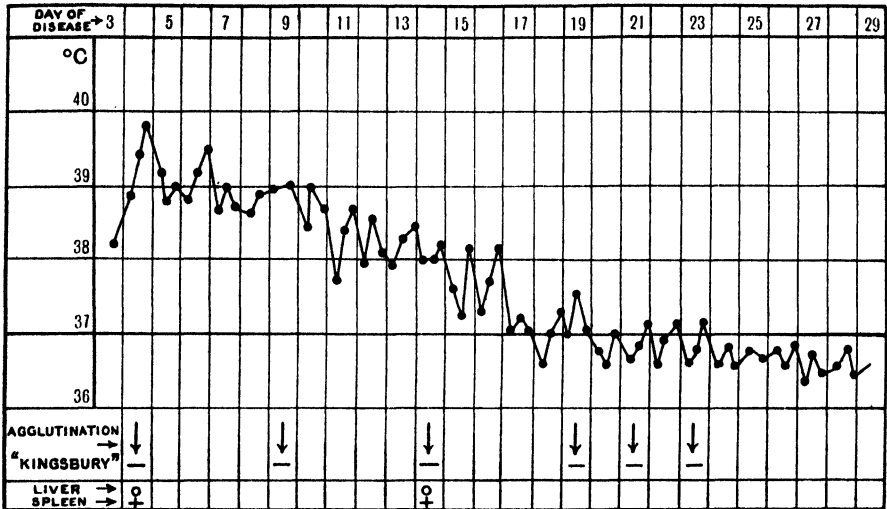


Chart III. No. 5061. Javanese labourer, ± 28 years. In hospital May 17th–June 24th, 1929. On admission fever, headache. Tsutsugamushi ulcer on left fossa inguinalis on the side of the scrotum with swollen inguinal glands. Widal negative. 5800 leucocytes. Cultures of blood, urine and faeces negative for *B. typhosus*. See chart for course of fever and Weil-Felix reaction.

#### DISCUSSION.

It has been shown that in thirty out of forty-five cases of tsutsugamushi disease a positive Weil-Felix reaction with the "Kingsbury" strain has been recorded. In many of these cases the agglutination reached a titre of 1 in 1000. The titre might even have been higher if further dilutions of the sera had been tested or suspensions of the O variants of the bacilli (Felix, 1930) had been used.

These facts support only the second statement quoted from Fletcher, namely, the close relationship between tsutsugamushi disease and scrub typhus. If further observations should reveal titres higher than 1 in 1000 occurring in cases of tsutsugamushi disease, the height of the titre will not form a useful method of differentiating these diseases. Perhaps the same objection might apply to the rather minute differences in the clinical course of these two diseases which are given by Fletcher and his co-workers as means of differential diagnosis. In all epidemics of infectious diseases fluctuations in severity of clinical symptoms occur and have been recorded of the same order as those observed in these diseases. Similar fluctuations are also to be seen in the case records published by Schüffner and Maasland<sup>1</sup>. In the present

<sup>1</sup> Even a primary ulcer was found only in a certain percentage of cases in the earlier descriptions of tsutsugamushi disease in Sumatra (78 per cent. out of 197 cases during 1915–21 and only in 59 out of the 156 cases described by Maasland). At that time Schüffner, having seen the primary ulcer in all his European cases, thought that its absence in some cases amongst native patients might be due to the fact that the ulcer was healed at the time of admission to the hospital. From the evidence provided by the more recent studies on tropical typhus it seems quite possible that some of the cases without ulcer might have been cases of so-called scrub typhus, that is if we regard the detection of a primary ulcer and bubo as the only valuable method of differentiation between tsutsugamushi disease and scrub typhus.



state of our knowledge of scrub typhus and tsutsugamushi disease it appears to me to be still an open question whether the two diseases are caused by different viruses or whether the same virus is involved in both diseases, but is spread by different vectors, of which the one does give a skin ulcer, and the other does not. In the case of scrub typhus, recent studies by Anigstein (1930) seem to make it possible to cultivate from the blood of patients strains of rickettsia-like organisms and to perform successful transmissions in guinea-pigs. Further experiments, combined with observations on immunity reactions in both diseases, may throw light on this question. In the meantime, for practical reasons, it seems advisable to separate tsutsugamushi disease from scrub typhus according to the presence or absence of the primary ulcer.

## SUMMARY.

1. The Weil-Felix reaction in a series of forty-five cases of tsutsugamushi disease (type Schüffner) is recorded.
2. The agglutination reaction was negative in fifteen cases, the other thirty showing a positive reaction with the "Kingsbury" strain of *B. proteus* X.
3. The Weil-Felix reaction is not regarded as an adequate method for differentiating tsutsugamushi disease from scrub typhus, since high titres (1 in 1000) occur in a considerable proportion of cases of tsutsugamushi disease.
4. In the present state of our knowledge of the two diseases it is recommended that they should be separated according to the presence or absence of a primary ulcer and bubo.

## REFERENCES.

- ANIGSTEIN, L. (1930). *Malayan Med. J.* **5**, 62.  
 BIEN, L. and SONNTAG, F. (1917). *Munch. Med. W.* **64**, 1409.  
 FELIX, A. (1930). *System of Bacteriology, Med. Res. Council*, **7**, 412.  
 FELIX, A. and OLITZKI, L. (1929). *Brit. J. Exp. Path.* **10**, 26.  
 FLETCHER, W. (1930). *Proc. Roy. Soc. Med.* **23**, 37.  
 FLETCHER, W. and LESSLAR, J. E. (1925). *Bull. Inst. Med. Res. Fed. Malay States*, No. 2.  
 FLETCHER, W., LESSLAR, J. E. and LEWTHWAITE, R. (1929). *Trans. Roy. Soc. Trop. Med. and Hyg.* **23**, 57.  
 ISHIWARA, K. and OGATA, N. (1923). *Centr. f. Bakt. Orig.* **1**, **90**, 164.  
 KAWAMURA, N. (1926). *Bull. Coll. Med. Univ. Cincinnati. Monograph.*  
 — (1930). *Kolle-Wassermann*, 3rd ed. **8**, 1387.  
 MAASLAND, J. H. (1926). *Thesis*, Amsterdam.  
 PRAUSNITZ, K. (1920). *Centr. f. Bakt. Orig.* **1**, **84**, 103.  
 SCHÜFFNER, W. (1909). *Geneesk. Tijdschr. v. Ned. Indië*, **49**, lxiv.  
 SCHÜFFNER, W. and WACHSMUTH, M. (1909). *Zeitschr. f. klin. Med.* **71**, Heft 1-2.  
 WEIL, E. and FELIX, A. (1917). *Wien. klin. Wochenschr.* **30**, 1509.  
 WOLFF, J. W. (1929). *Geneesk. Tijdschr. v. Ned. Indië*, **69**, 429.

## EPIDEMIC DYSENTERY IN THE NURSING STAFF DUE TO *BACILLUS DYSENTERIAE* (SONNE).

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(With Plate VI.)

AN extensive outbreak of a mild dysenteroid nature occurred among the nursing staff at Guy's Hospital during February 1930. About one hundred of the staff were affected.

It is of extreme interest and at the same time very gratifying to note that, although many affected nurses continued their duties in the wards, no single case was observed among the hospital patients. This suggests that the scrupulous cleanliness of the nurses was sufficient precaution against the spread of infection.

There was no difficulty in establishing the *Bacillus dysenteriae* (Sonne) as the causal organism; and the evidence, although not conclusive, pointed to infection of food in the kitchens by a carrier.

The majority of recorded epidemics have been of this mild nature—acute diarrhoea and toxic symptoms usually followed by a rapid and complete recovery except in children who may die from the toxæmia.

*B. dysenteriae* (Sonne) is now a well-defined organism which, although first satisfactorily established as pathogenic by Sonne in 1915, had been previously isolated by other investigators who either failed to realise its significance or, owing to unobserved delayed fermentations of lactose and litmus milk, placed it amongst the Flexner Group as an inagglutinable strain. It has been shown to have an extensive distribution in Scandinavia, but was not definitely recognised in the British Isles until 1924, when Smith (1924) described a small outbreak in Aberdeen affecting four children. Since this date a steadily increasing number of isolated cases and epidemics have been recorded and, according to Gardner (1930) and a personal communication from Dr W. M. Scott of the Ministry of Health, there has been a much larger number of unrecorded outbreaks throughout Great Britain.

The evidence would suggest that the organism has travelled from Scandinavia viâ Scotland to England unless it is presumed that it had previously been endemic but undetected. Some support is given to the latter hypothesis by Nabarro (1927), who claims that bacilli which he isolated in 1921 from cases of summer diarrhoea and called "inagglutinable *B. Flexner*" were identical with *B. dysenteriae* (Sonne). At the same time he protests against

separate names being given to such organisms of the Flexner group because of slight differences in biological characters, and suggests the group name of *B. coli dysenteroides*. We contend, however, that the sharply defined serological reactions and the extensive clinical distribution of this organism are ample reasons for a separate name.

Epidemics and cases have now been described in Australia, Brazil, Denmark, Egypt, France, Germany, Japan, India and U.S.A.—a world-wide distribution.

The incidence rate is difficult to assess, as in an epidemic many infected individuals are not sufficiently ill to deem it necessary to seek medical advice.

#### THE OUTBREAK.

One isolated case (Nurse E.) occurred 15. ii. 30, 5 clear days before the main wave. This nurse had more severe symptoms than the majority. It was thought that she might in some manner have been responsible for the outbreak, and a careful enquiry was made into her history. As far as could be ascertained she had had no contact with any illness of this nature, but in January (6 weeks previously) she had had diarrhoea and vomiting for 3 days. There was then no pyrexia, and the patient stated that the attack was in no way comparable to her present illness. In any case it was impossible to find any likely method by which she could have contaminated food used by the other nurses either directly or indirectly.

The main wave, estimated at about one hundred cases, occurred within the next 7 days (20–28. ii. 1930), but only half of these reported sick.

No cases, except the two recorded below, could be described as severe; the onset was commonly marked by giddiness and malaise, followed in a few hours by abdominal discomfort and diarrhoea. Pyrexia, if present, was never higher than 101° F., falling to normal within 48 hours.

Two cases occurred a few days after the main wave and both showed unusual and severe symptoms.

#### (1) *Sister M.*

The general toxæmia was severe and the illness was accompanied by an excruciating headache. Although the organisms rapidly disappeared from the stools, convalescence was slow.

#### (2) *Nurse B. W.*

This patient became ill on March 4th, with diarrhoea, but little constitutional disturbance and no pyrexia. Her stools contained a little blood and mucus for the first week, but we failed to isolate Sonne's bacillus. Examinations for other pathogenic organisms or amoebae were also negative. The diarrhoea persisted, on the average about four stools being passed per diem. Repeated examinations of the stools were negative. After 5 weeks, during which she had been completely apyrexial, the temperature commenced to show slight

evening rises to 99° or 99.5° F., and the frequent motions continued. At the end of two more weeks (7 weeks from the onset) the condition was unchanged, and it was decided to give anti-dysenteric serum (B. W.'s polyvalent). Four intramuscular injections of 5000 units were given on consecutive days. These injections produced neither reactions nor apparent change in her condition. Seven days later a fifth injection of 5000 units was given. Within 48 hours the temperature had become normal and remained so, although the number of motions did not decrease for another 2 weeks, when the patient made a rapid recovery.

A proctoscopic examination made late in the course of the illness showed granular changes, but no ulceration of the mucosa.

It is debatable whether this was an infection with *B. dysenteriae* Sonne, as the illness differed so much from that in the other cases. At no time was a known pathogenic organism isolated from the stools and no agglutinins to Sonne, Shiga or Flexner bacilli were found in her blood.

The part played by the anti-dysenteric serum in her recovery is doubtful.

#### INVESTIGATIONS.

Laboratory investigations were commenced on February 24th, and by this time a considerable number of cases were well on the way to recovery. Attempts were made to secure stools from all known infected persons, and blood was taken for agglutination. As will be seen in the bacteriological notes *B. dysenteriae* Sonne was isolated from sixteen cases out of forty-two examined. About 30 per cent. of the cases showed a small amount of blood and mucus in the early motions.

In all investigated cases *B. dysenteriae* Sonne had disappeared from the stools within a few days.

Full details of the organisms and agglutinins present are given below.

#### DISCUSSION OF CAUSE.

As soon as the nature of the epidemic was appreciated investigations into the possible cause were commenced. After discussion with the matron and sisters it became clear that all cases had occurred solely in persons who fed in the nurses' dining room. The sisters have a separate dining room, supplied from a different kitchen, and only those sisters who supervised the nurses' dining room and had their meals there were affected.

No article of food or drink was purchased and/or supplied solely to this kitchen. The large number of cases, and their occurrence over a period of 14 days, made it impossible to suspect that only one part of the milk or food supply contained the organism on arrival at the hospital.

Another possible source of infection was the water supply. This, unfortunately, was not investigated. Again, however, it would be difficult to explain the limitation of infection.

It was therefore presumed that infection took place during the preparation

of food not previously contaminated. Stools and blood were therefore obtained from every one who handled or came in contact with the food in the kitchen, when it was found that two kitchen maids gave positive cultures from the stools and one showed agglutinins in the blood. Neither had been ill and a careful enquiry into their history and contacts for the last 6 months failed to disclose any illness or contact with illness that could be interpreted as a Sonne infection.

These two maids were removed and placed in the Isolation Ward. No further cases occurred after this step, but it is only fair to note that the epidemic was already showing signs of exhaustion or intermission.

The faeces of the two kitchen maids became negative in 3 days and, upon repeated negative examinations, they were allowed to return to work.

It must be concluded that, despite the rapid disappearance of the organism from the stools, one or both of these maids infected the food.

#### A CASE OF LABORATORY INFECTION.

During the bacteriological investigations one of the laboratory assistants (F. M.) who was employed exclusively on this work became infected from a culture; he had a typical illness lasting a week, followed by complete recovery. *B. dysenteriae* (Sonne) was recovered from his stools.

#### BACTERIOLOGICAL NOTES.

The following investigations were carried out on the sixteen strains of Sonne's bacillus isolated from the forty-two cases investigated, as well as one strain isolated from a laboratory infection, and one strain from the intestinal mucosa of a rabbit which died as the result of artificial feeding with cultures of the organism.

##### *Isolation.*

All specimens of faeces were plated out on nutrose litmus agar—the Guy's Hospital modification of Conradi-Drigalski. After 24 hours' incubation at 37° C., non-lactose fermenting colonies were picked and planted on to Andrade's indicator agar (phenol red), thence into "sugars" if no change occurred.

In all positive cases the number of non-lactose fermenting colonies on the nutrose plates was fairly large, forming more than 20 per cent. of the total number of colonies. In about half the cases more than 50 per cent. were non-lactose fermenters, while, in three, Sonne's bacillus was present practically in pure culture. Provided specimens are received in the first 24 hours of the disease, there is no difficulty in isolating the organism. It soon becomes rare, however, and in only one case was it present longer than 3 days after the onset of symptoms.

##### *Colony appearances.*

All plates showed two types of colony, which on macroscopic appearances could be described as rough and smooth, the latter, however, being far more

numerous. Smooth colonies were somewhat larger and more opaque than typical Shiga or Flexner colonies, while the rough were much larger, flatter and with spreading serrated edges. (See Plate VI, figs. 1-3.)

There is a distinct sweetish smell about the cultures resembling that noted in culture of *B. dysenteriae* Shiga, which has been described as spermatic.

*Fermentation reactions.*

Both rough and smooth colonies were used in order to ascertain if any difference in fermentative activity existed between the two forms. There was marked permanent acidity in litmus milk. Indol was not produced in any case.

Table I. *Fermentation reactions of the smooth and rough types isolated from each individual case.*

Case no.	Type of colony	Lactose	Dextrose	Maltose	Saccharose	Mannite	Glycerin	Inulin	Arabinose	Litmus milk	Indol
1	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
2	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
3	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
4	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
5	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
6	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
7	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
8	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
9	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
10	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
11	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
12	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
13	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
14	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
15	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
16	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0
17	S.	+	+	+	+	+	0	0	+	+	0
	R.	+	+	+	+	+	0	0	+	+	0

+ = Acid reaction. 0 = No change. The figures in parentheses denote the day of culture on which the acid reaction was first observed.

It is generally accepted that the rough form of this organism has more active fermentative powers than the smooth. As will be seen, however, from Table I, the variation in lactose was in no way consistent. In the majority of strains there was a difference in the time taken for acid production, but as frequently as not the smooth showed the shorter interval. In the case of saccharose there is a definite lag on the part of the rough in producing acid.

*Diagnosis.*

As lactose takes some days to be fermented, it is unsatisfactory to await a diagnosis on the fermentation reactions alone.

The diagnosis was made by agglutination of the organism with a specific serum kindly supplied to us by Dr W. M. Scott. This serum agglutinated all strains up to a titre of 1 in 1000.

Other sera, Flexner (V, W, X, Y and Z) and Shiga, failed to agglutinate even in dilutions of 1 in 20. As cultures of rough forms failed to agglutinate with our original serum, only smooth colonies were used for diagnosis. This serum was presumably prepared from smooth strains only.

*Presence of agglutinins in blood of infected cases.*

In order to ascertain to what extent, if any, agglutinins were produced to smooth and rough varieties, the sera of infected cases were put up against their own organisms, both smooth and rough forms isolated from the original stool. The macroscopic method of agglutination was used, readings being taken after 3 hours in the water bath at 56° C., and again after 24 hours at room temperature. As agglutination of non-flagellated organisms of the colon-typhoid group is of the fine granular variety, it was found convenient to read results in a concave mirror, as the finest agglutination could thereby be seen and any difference between smooth and rough forms noted. For dilution purposes 0.9 saline was used, and here it must be mentioned that there was no evidence of salt sensitivity of the rough forms, a point which was investigated and will be discussed later on. The agglutination of the rough variety was indistinguishable from that of the smooth and there was no evidence of spontaneous agglutination in the control tubes.

*Notes on smooth and rough variations.*

It was thought that further investigations into the agglutinogenic properties of the two colonial forms would not be without interest, so it was decided to carry out further experiments. We use the term "variation" and "variants," not because there is proof that these forms are true bacterial variants, but for lack of a better descriptive term. This doubt exists in the case of our rough colonies which, as we shall see, do not fulfil all the requirements of the true rough variant.

Smooth and rough colonies were picked off on to agar plates and subcultured every 24 hours until apparently pure cultures of both forms were obtained. The rough forms were easily separated and maintained in pure culture, and have shown no tendency to revert to a smooth type. The smooth colonies, however, gave rise to a few rough colonies, which soon disappeared after five or six subcultures, and these too have maintained their form for at least 2 months, but eventually rough forms reappeared. Each form

was then seeded into 100 c.c. of nutrient broth, incubated for 24 hours at 37° C., and then heated in the water bath at 60° C. for 1 hour and sufficient formalin added to make a concentration of 0.2 per cent. This was done to ensure stable suspensions for agglutination purposes. The smooth forms gave a uniform turbidity in broth while the rough forms completely settled down, leaving a clear supernatant medium which, on shaking, however, became uniformly turbid.

This sedimentation of the rough form is in no way akin to agglutination as no clumping, even of the fine granular variety, could be seen microscopically, and this is confirmed when the rough suspension is agglutinated by an appropriate rough serum, when true agglutination takes place and clumping is obvious.

*Analysis (see Table II).*

Four patients showed no demonstrable agglutinins of either kind (Nos. 7, 8, 10 and 17), four agglutinins to the smooth variant only (Nos. 4, 11, 12 and 15),

Table II. *Agglutination of the smooth and rough variants by the respective homologous human sera.*

Case no.	Type of colony	Titre of serum						Control
		1/20	1/40	1/80	1/100	1/150	1/200	
1	S.	+	+	+	±	-	-	-
	R.	+	+	+	-	-	-	-
2	S.	+	+	-	-	-	-	-
	R.	+	+	-	-	-	-	-
3	S.	+	-	-	-	-	-	-
	R.	+	+	±	-	-	-	-
4	S.	+	±	-	-	-	-	-
	R.	-	-	-	-	-	-	-
5	S.	-	-	-	-	-	-	-
	R.	+	±	-	-	-	-	-
6	S.	+	+	+	+	±	-	-
	R.	+	-	-	-	-	-	-
7	S.	-	-	-	-	-	-	-
	R.	-	-	-	-	-	-	-
8	S.	-	-	-	-	-	-	-
	R.	-	-	-	-	-	-	-
9	S.	-	-	-	-	-	-	-
	R.	+	+	-	-	-	-	-
10	S.	-	-	-	-	-	-	-
	R.	-	-	-	-	-	-	-
11	S.	+	+	-	-	-	-	-
	R.	-	-	-	-	-	-	-
12	S.	+	+	±	-	-	-	-
	R.	-	-	-	-	-	-	-
13	S.	+	+	+	+	-	-	-
	R.	+	±	-	-	-	-	-
14	S.	+	-	-	-	-	-	-
	R.	+	+	±	-	-	-	-
15	S.	±	-	-	-	-	-	-
	R.	-	-	-	-	-	-	-
16	S.	+	+	+	-	-	-	-
	R.	+	+	-	-	-	-	-
17	S.	-	-	-	-	-	-	-
	R.	-	-	-	-	-	-	-

+ = Complete agglutination. ± = Partial agglutination. - = Absence of agglutination.



two to the rough variant only (Nos. 5 and 9), while seven showed agglutinins to both forms (Nos. 1, 2, 3, 6, 13, 14 and 16).

The most noticeable features shown are:

1. Agglutinins may be absent altogether in some cases.
2. When present, they are so in low titre. This may be due to the fact that in this epidemic, the disease was of a very mild type with little toxæmia and therefore little antibody response.
3. Agglutinins to the smooth form have a tendency to be more common and in greater amount.
4. Agglutinins may be present for both varieties at the same time. This last feature needs discussion as it presents possibilities which are difficult to understand. That there should be agglutinins in the blood to both forms would seem to indicate that both smooth and rough forms have invaded the intestinal mucosa. Both rough and smooth colonies were present on the original plates from the faeces, therefore both forms were simultaneously present at the time of the invasion. As will be seen later, there is little difference in the virulence between the two forms, therefore it is reasonable to suggest that their agglutinogenic properties do not vary widely. This also is confirmed by subsequent experiments. It is most regrettable that insufficient serum was obtained to allow absorption tests to be carried out as these would have helped to confirm our opinion that agglutinins of both varieties are due to infection or invasion of the intestinal mucosa with both variants. That either is capable of producing antibodies to itself alone is borne out by the fact that agglutinins of one type alone may be present. The practical bearing that this has lies in the fact that both smooth and rough suspensions should be used when attempting to demonstrate agglutinins in the blood of Sonne infections, as otherwise a number of sera containing rough agglutinins only would be missed.

*Production of specific agglutinins to smooth and rough forms in rabbits.*

Twenty-four hour agar cultures were used. Saline suspensions containing 500 million organisms per c.c. were heated for 1 hour at 60° C. and 1 c.c. was injected intravenously at weekly intervals for 3 or more weeks, three injections being usually sufficient to produce titres of 1 in 2000. In this way smooth and rough anti-sera were produced without difficulty. In order to produce a serum containing both smooth and rough agglutinins 0.5 c.c. of each suspension was injected simultaneously. The results of subsequent agglutination are shown in Tables III, IV. Each serum was put up against both smooth and rough suspensions.

*Agglutination by specific sera.*

Serum dilutions were used in the following strengths of 1/20, 1/40, 1/80, 1/100, 1/500, 1/1000, 1/2000 and 1/3000. The smooth type was agglutinated by the smooth serum up to 1/2000, while the rough organisms were not agglutinated in any dilution. The rough serum agglutinated the rough organism

[illegible]

Absorption with rough organisms was more successful than absorption with smooth, as the latter suspensions were still agglutinated to a third of their original titre. At the same time the titre for rough suspensions was reduced to some extent, though this remained higher than that for smooth. In the case of absorption with rough organisms, however, all rough agglutinins were absorbed successfully, though here again the titre for smooth suspension was reduced.

Why absorption with rough forms should be more successful is difficult of explanation, unless there is greater affinity between rough agglutinins and their respective organisms than that existing between the smooth varieties at 37° C. which was the temperature used during absorption. The series is too small a one on which to base any definite conclusions and the titre of the sera insufficiently high. These somewhat anomalous results led us to investigate the question whether we were dealing with rough variants in the strict sense of the word. With this object in view it was decided to treat our rough cultures with alcohol and chloroform by the method advocated by White (1927).

Fifteen c.c. of absolute alcohol were added to each plate of rough cultures and the "suspensions" heated for 30 minutes in the water bath at 56° C., after which 10 c.c. of chloroform were added and the "suspension" left at 56° C. for 4 hours with frequent shaking. The organisms were then centrifugised, washed and re-washed in alcohol and suspensions made up to 2000 millions per c.c.

As all our organisms were of one serological strain, only three were picked out at random and treated thus. These three rough bacilli, Nos. 4, 5 and 6, which previously were agglutinated by the rough serum up to a titre of 1 in 2000 were now not agglutinated. As a control the corresponding smooth cultures were similarly treated and this caused all agglutination to disappear. According to White the smooth variant should retain its agglutinability after such treatment. Our contradictory result invalidated the differential value of the test. This and the absence of salt sensitivity are the only two arguments against this particular rough strain of Sonne being a typical rough variant. On the other hand, the colonial appearances, the serological reactions and the pathogenicity for rabbits suggest strongly that this particular strain is a true rough variant. Of course, one could postulate an intermediate form showing some characters and not others, but in our opinion such an assumption, in view of the lack of sufficient data on the serology of Sonne infections, particularly of the two types of agglutinins referred to in the literature, is unwarranted.

#### *Pathogenicity for rabbits.*

Feeding experiments were first tried. A batch of four rabbits were fed on bran with which had been mixed living broth cultures of the smooth form of Sonne's bacillus, four more were fed on rough cultures and four kept as controls. Successful infection was not expected as the rabbits' gastric juice was thought to be sufficiently lethal to bacteria to prevent infection. One of

the number fed on smooth cultures, however, became ill, severe diarrhoea developing on the second day after feeding and death resulting on the fifth day. At autopsy the whole of the colonic mucosa was acutely inflamed and haemorrhagic, the peritoneum was injected, but all the other serous cavities appeared normal. Sonne's bacillus was recovered from the intestinal mucosa, but cultures from the heart and from the peritoneum remained sterile. All the other animals remained well.

As far as we can ascertain this is the first case of spontaneous infection of a rabbit during feeding experiments. Bamforth (1924), Kerrin (1928), and Ray (1930), have made attempts to do so without any successful case of infection.

Intravenous injections produced results very similar to those noted when *B. dysenteriae* (Shiga) is employed. Thus 2000 million dead bacilli generally caused severe diarrhoea and wasting in 24 hours and death in 4 to 8 days. Four rabbits injected with rough suspensions died after one injection and two more after the second injection a week later. Three out of six infected with a similar dose of smooth suspensions died, and three more after a third dose at weekly intervals. Doses of 500 to 1000 millions of dead bacilli of either smooth or rough forms caused diarrhoea with wasting of varying severity which sooner or later cleared up. On the whole there is little difference between the two forms, if anything, the rough form was found to be slightly more toxic.

#### CONCLUSIONS.

1. Diagnosis depends primarily on finding the organism in the stools and agglutination with a specific serum. Both rough and smooth sera should be employed for their respective variant.
2. High titre agglutinating rough serum can be produced with as equal ease as a similar smooth serum.
3. In our series the patients' sera showed agglutinins present only in low titre, and in some cases they were absent altogether. Two cases had agglutinins to the rough organism only. A negative result is of little diagnostic value.
4. The smooth and rough variations of *B. dysenteriae* (Sonne) require further investigation, especially the agglutinogenic properties of each variant.

#### SUMMARY.

1. An epidemic of a dysenteroid nature, largely ambulatory, due to *B. dysenteriae* (Sonne), affecting some 100 nurses without transference to any hospital case in the wards served by this section of the nursing staff, is described.
2. Sonne's bacillus was isolated from sixteen out of forty-two cases examined.
3. The probable cause was infection of food during preparation in the kitchens by a carrier.
4. A case of infection from laboratory cultures is described.

5. Notes are given of investigations upon the colonial appearance, biochemical and agglutinogenic reactions and pathogenicity to rabbits of the organism isolated, and also upon the presence of agglutinins in the blood of infected cases.

6. One case of infection in a rabbit by feeding experiments is described.

We would express our sincere thanks to Prof. J. Eyre for his advice, especially with the preparation of this paper, and to Dr W. M. Scott of the Ministry of Health for assistance in identification and the supply of agglutinating sera.

#### REFERENCES.

- BAMFORTH, J. (1924). *Proc. Roy. Soc. Med. London*, **17**. *Sect. Trop. Dis. and Parasit.* **25**, 29.  
GARDNER, A. D. (1930). *A System of Bacteriology* (Medical Research Council), **4**, 244.  
KERRIN, J. C. (1928). *J. Hygiene*, **28**, 4.  
NABARRO, D. (1927). *J. Path. and Bact.* **30**, 176.  
RAY, HILDA (1930). *J. Hygiene*, **30**, 25.  
SMITH, J. (1924). *J. Hygiene*, **23**, 94.  
SONNE, C. (1915). *Centralbl. f. Bakt.* **76**, 65.  
WHITE, P. BRUCE (1927). *J. Path. and Bact.* **30**, 113.

#### EXPLANATION OF PLATE VI.

*Bacillus dysenteriae* (Sonne). Colonies in plate cultures.

Fig. 1. Rough type of colonies.

Fig. 2. Mixed rough and smooth types of colonies in first subculture from faeces.

Fig. 3. Smooth type of colonies.

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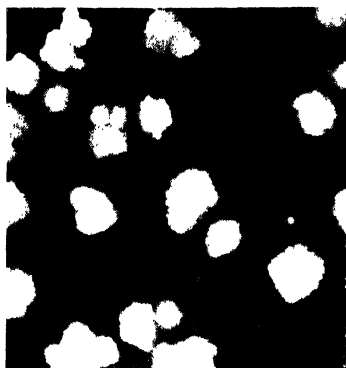


Fig. 1

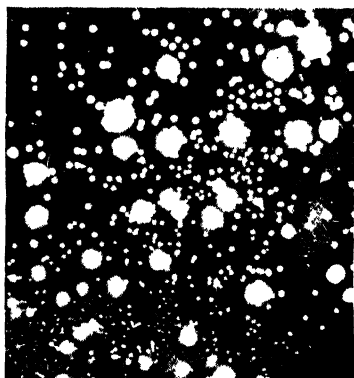


Fig. 2

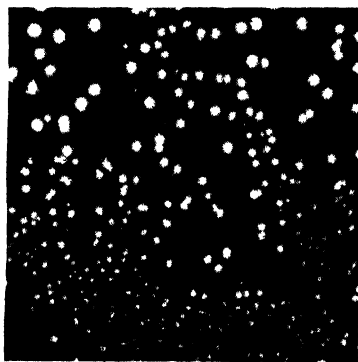


Fig. 3



## KEEPING QUALITY OF MILK AND THE AGE ON TESTING FOR TOTAL BACTERIAL COUNT.

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DURING the period 1923-29 inclusive nearly six thousand samples of milk were tested at Wye for both total bacterial count and also keeping quality. An examination of the results shows that on the average the afternoon milks showed *nine hours less* keeping quality than morning milks of the same total bacterial count, see Table I. The age of the milk on testing for total bacterial count (reckoned from the time of milking) is 27-29 hours for morning milks and 20-24 hours for afternoon milks.

According to Table I the average keeping quality for samples of afternoon

Table I. *Keeping quality of morning and afternoon milks.*

Colonies per c.c.	Morning milk Av. K.Q.*	Afternoon milk Av. K.Q.*
0- 500	87.7	77.9
500- 1,000	83.7	73.9
1,000- 2,500	81.0	71.8
2,500- 5,000	78.0	67.2
5,000- 7,500	75.2	63.4
7,500- 10,000	71.4	63.8
10,000- 20,000	70.2	60.0
20,000- 30,000	68.0	59.9
30,000- 50,000	66.8	55.8
50,000-100,000	62.1	52.9
100,000-200,000	57.2	51.6
200,000-500,000	54.0	47.8
500,000-750,000	54.2	45.2
Over 750,000	—	—
Total of samples tested	2,808	3,032

\* Av. K.Q. stands for average keeping quality in hours.

milk with a total count of 1,000-2,500 per c.c. (380 samples) is 71.8 hours, and this is the same average as shown by samples of morning milk where the total count is 7,500-10,000 per c.c. (146 samples). Similar comparisons could be made over the whole range of total count. It might be argued that a similar average keeping quality should imply a similar average total count. This would mean that samples of afternoon milk giving a total count of 1,000-2,500 per c.c. when tested at 24 hours old should, if tested at 28 hours old, record a total count of 7,500-10,000 per c.c. in order to be on an equal footing with samples of morning milk.

To investigate this, 258 samples of afternoon milk were tested for total count when 24 hours old; they were then kept for four hours at 60° F. and re-tested for total count when 28 hours old. The work was spread over two



years. Very little change in count took place in the four-hour interval. The results are summarised in Table II.

The results were arranged in count groups of 0 to 500 per c.c., 500 to 1,000 per c.c. and so forth, considering the total count when 24 hours old. The average keeping quality of each count group was thus obtained. The 258 results were then re-arranged in the same count groups, but in accordance with the total count when tested at 28 hours old. A fresh series of keeping-quality averages was thus obtained. Finally it was possible to compare the average

Table II. *Duplicate tests afternoon milk 24 and 28 hours old.*

Colonies per c.c.	24 hour tests		28 hour tests	
	No. of samples	Av. k.q.*	No. of samples	Av. k.q.*
0- 500	54	75.9	58	75.1
500- 1,000	36	66.9	35	65.2
1,000- 2,500	55	64.8	47	66.3
2,500- 5,000	31	64.7	27	64.8
5,000- 7,500	12	64.8	13	66.9
7,500- 10,000	6	61.3	5	64.0
10,000- 20,000	22	59.4	19	65.1
20,000- 30,000	10	58.0	9	58.8
30,000- 50,000	9	56.7	15	55.6
50,000-100,000	6	55.0	7	51.3
100,000-200,000	9	55.5	5	53.9
200,000-500,000	7	50.0	8	49.8
500,000-750,000	1	47.5	3	56.6
Over 750,000	—	—	7	50.6
Total of samples tested	258	—	258	—

\* Av. k.q. stands for average keeping quality in hours.

keeping quality given by the experimental samples both at 24 hours old and at 28 hours old (Table II) against the averages obtained by the routine samples over a number of years (Table I).

Despite the comparatively small total of experimental samples it will be seen that the average keeping quality at 24 hours old and at 28 hours old is practically the same, and that both these series of figures agree very closely with the figures for afternoon milks in Table I. There is no indication here that the nine-hour difference in keeping quality is connected with the four-hour difference in the age on testing.

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# DÖDERLEIN'S VAGINAL BACILLUS: A CONTRIBUTION TO THE STUDY OF THE *LACTO-BACILLI*.

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(With 1 Text-figure.)

THIS study of the characters of Döderlein's bacillus is offered as a supplement to an investigation into the vaginal flora of women during pregnancy (Cruickshank and Baird, 1930). Smears and cultures were made from the vaginal swabbings of 200 pregnant women, more than half of whom were examined at regular intervals from the third month of pregnancy until term. Several cultures of Döderlein's bacillus could thus be recovered from the vagina of the same woman. Three types of bacterial flora were recognised according to the character of the direct smear—"Grade A," composed entirely of Döderlein's bacillus and representing the normal flora; "Grade B," a mixture of Döderlein's bacillus and diphtheroids; and "Grade C," consisting of a profuse bacterial mixture in which diphtheroids, enterococci and staphylococci were most numerous.

No systematic study of Döderlein's bacillus has been reported in this country and the descriptions of the organism given by German and American workers have been mostly derived from the study of a small number of strains (Jötten, 1922; Rother, 1922; Lash and Kaplan, 1926; Thomas, 1928). A review of the literature is given elsewhere (Cruickshank and Cruickshank, 1930). The difficulty of isolating Döderlein's bacillus and maintaining it in culture has been stressed as the stumbling-block to an adequate study of the organism. In the present investigation the primary isolation of the organism has not presented much difficulty, but its subsequent maintenance is not easy and many strains could not be propagated: of over 200 strains originally isolated, 60 have been studied more or less fully, but only 20 of these 60 strains are still alive after 9-18 months' sub-culture. The group of lacto-bacilli to which Döderlein's bacillus belongs forms peroxide without catalase, and like other organisms with like characters, *e.g.* *Pneumococcus* and *S. viridans*, is difficult to maintain on artificial culture media (see McLeod and Gordon, 1922, 1923).

## ISOLATION.

Cultures were made within 2-3 hours after the vaginal swab was taken. The routine procedure adopted for the isolation of Döderlein's bacillus was to smear the swab over the surface of a 4-inch Petri plate containing one per cent. lactose or glucose hormone agar *plus* 5 per cent. defibrinated rabbit blood and to incubate the plate aerobically for 48 hours at 37° C. If the stained smear showed a "Grade A" flora and the swab was cleanly taken, without vulvar contamination, the result was usually a pure growth of Döderlein's bacillus. If the

flora was mixed, Döderlein's bacillus was much less easily isolated, although if present the colonies, being smaller and less opaque, could readily be distinguished from those of diphtheroids or enterococci. Single colonies were sub-cultured on to serum agar which was used for the subsequent maintenance of the organism. At first sub-cultures were made every second day and later once a week. For the initial isolation a fermentable sugar seems to be the most essential addition to the medium; whole blood is an adjuvant and probably acts as a catalyst to destroy peroxide. Anaerobiasis is not necessary despite statements to the contrary although growth seemed sometimes to be accelerated by cultivation in an atmosphere of reduced oxygen tension. Primary culture in an acidified fluid medium is less useful than it is for the isolation of the intestinal *B. acidophilus*. Occasionally no growth of Döderlein's bacillus was obtained on lactose blood agar when incubated aerobically despite its presence in apparently pure culture in the direct smear. Frequently the first sub-culture from lactose blood agar to serum agar was unsuccessful. The addition of 0.1 per cent. glucose to the serum agar might aid growth at this stage and in subsequent sub-culture.

#### MORPHOLOGY.

Döderlein's bacillus, like the other lacto-bacilli, is as a rule sufficiently distinctive morphologically to permit a tentative identification from a Gram-stained smear. Non-motile, non-sporing, non-capsulated Gram-positive rods of varying length and thickness and usually slightly curved, they show a lack of rigidity and have a typical grouping which differentiates them from *B. subtilis* on the one hand and diphtheroids on the other. *B. diphtheriae* in a Gram-stained film may, however, closely resemble Döderlein's bacillus. Among 60 strains studied, three main morphological types were recognised: *Type a*, most common—about 70 per cent. of the whole group—is rather slender, slightly curved and of varying length with, even in young cultures, a fair proportion of longer forms some of which tend to lose Gram's stain. The bacilli are arranged in twos and threes forming obtuse angles and Y-shaped figures, or in bundles or palisades, rather like the grouping of *B. diphtheriae* (Fig. 1). There is no chain formation, but after a few days' growth long curling bacilli become numerous and form tangled skeins. *Type b*, the second in order of frequency, is a short plump coccobacillus, either straight or slightly curved and intensely Gram-positive; there is less morphological uniformity than in the first type, a small proportion of long slender curling forms being present alongside the short thick individuals (Fig. 2). Short chains of three or four are found and clumps of the shorter forms occur. *Type c* is a streptobacillus, there being long chains of slender even-sized bacilli, and whorled masses of the organisms like those of the anthrax bacillus (Fig. 3). Only 5 out of the 60 strains were of this type. In addition, there were strains not exactly conforming to any of these three representative types, e.g. a short straight diphtheroid-like organism, a long thick curling type the individual members being like bits of elastic, and one unusual strain with a bulbous central

portion (Fig. 4). Notes were made of the morphological appearances of each strain when first isolated and most of them have maintained their individual

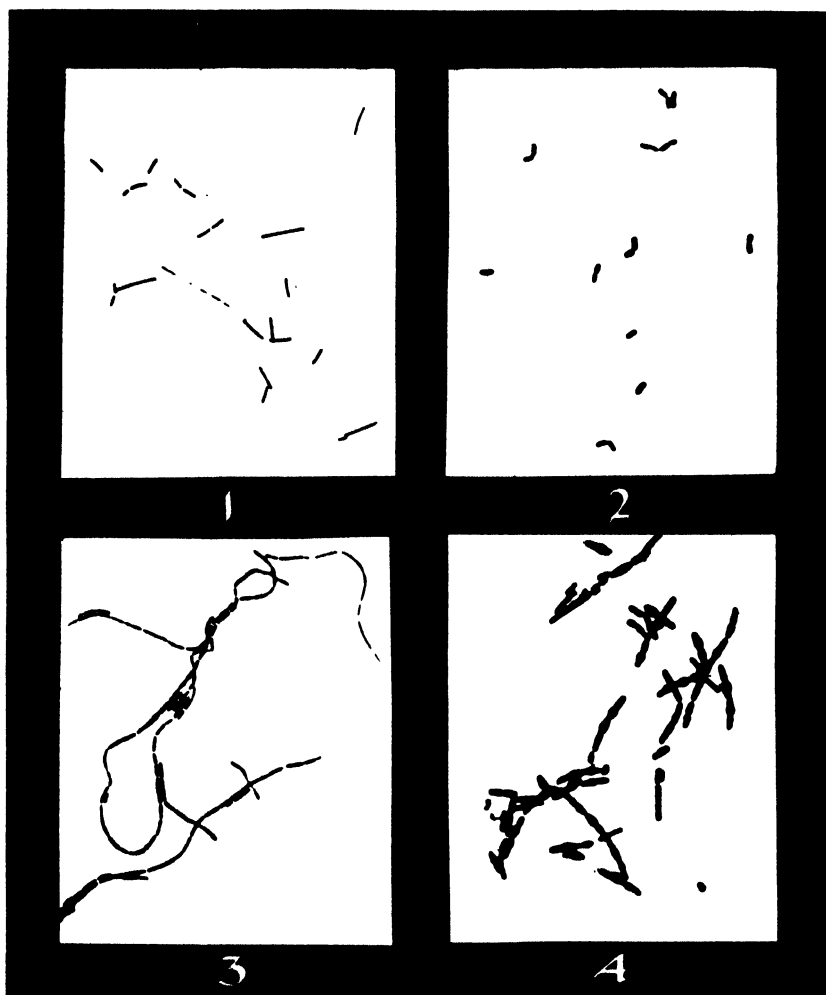


Fig. 1. *Type a*, the commonest, with grouping recalling that of *B. diphtheriae*.

Fig. 2. *Type b*, the next in frequency. Short thick individuals.

Fig. 3. *Type c*, long chains recalling those of *B. anthracis*.

Fig. 4. An unusual strain with central bulbous swelling.

characters 6–12 months after isolation. In older cultures there is an increasing pleomorphism—long curving threads, granular staining, bulbous extremities, and a tendency to lose Gram's stain.

#### CULTURAL CHARACTERS.

Surface colonies usually require 48–72 hours to develop fully; the growth of certain strains, even after prolonged sub-culture, is visible only after 48 hours' incubation. Individual colonies are at first very small, discrete, glistening and translucent. On the 3rd–4th day they are 1–2 mm. in diameter, slightly raised to the centre of the colony, translucent, with a matt surface

and a granular appearance by transmitted light; they have as a more characteristic feature a spreading irregularly crenated periphery. This colony appearance is similar to that of other varieties of lacto-bacilli and was common to most of the strains of Döderlein's bacillus irrespective of morphological type. The short plump forms, however, tended to grow as a more compact colony with an even well-defined margin. Colonies of diphtheroids or enterococci were bigger and more opaque. In poured plates of hormone or glucose agar, the deep colonies of Döderlein's bacillus were again characteristic of the lacto-bacillus group, the crab colony of *B. acidophilus* Y-type being a common form, *i.e.* a fairly compact colony with ragged pseudopodial outgrowths around it; others were smoother, round or lenticular, with one or two lateral buds, like the deep colony of *B. bifidus*: the delicate "feathery" colony was less common, although gradations from the "crab" to the "feathery" type of colony were frequently seen. A description (with illustrations) of the types of colony formation of Döderlein's bacillus is given by Jötten (1922).

On blood agar in primary isolation there was frequently a brownish discoloration of the medium and occasionally a greenish coloration was noticeable especially round individual colonies when these were sufficiently separate. The green-pigment formation, due possibly, as McLeod suggests, to the action of formed peroxide on haematin, was best demonstrated when sub-cultures were made on boiled blood agar—10 per cent. defibrinated rabbit blood in hormone agar heated until the medium became chocolate-coloured, and used freshly. Most of the strains were vigorous producers of peroxide, as judged by the extent and brightness of the green pigment (equivalent to *S. viridans*), some were less active, causing only a dull greenish coloration. Several strains regarded with suspicion because of their heavy growth and diphtheroid appearance were classified as diphtheroids when they failed to produce the green pigmentation. This property of peroxide production without catalase which is shared by strains of lacto-bacillus from stomach, intestines and mouth, may be regarded as an important distinguishing character of the group. The oxidative power of Döderlein's bacillus was demonstrated by pouring over a plate culture of the organism a solution of dimethyl phenylene-diamine for which I am indebted to Professor J. W. McLeod: the tetramethyl compound did not give satisfactory results (see Gordon and McLeod, 1928).

The occasional appearance of colonies of Döderlein's bacillus on MacConkey's bile salt lactose agar after plating non-catheter specimens of urine from female patients showed that the organism could grow on a medium of low-surface tension. It has been claimed that this property may help to differentiate *B. acidophilus* from *B. bulgaricus* (Albus and Holm, 1925) and explain why the former but not the latter can be implanted in the intestine. In the present study, strains from carious teeth, from stomach and intestine, as well as Döderlein's bacillus, were plated on 1 per cent. lactose agar containing 0.5 sodium taurocholate (commercial bile salt). All the strains tested grew on this medium, the teeth and stomach strains most vigorously as they do on other media. *B. bulgaricus* was not tested.

## BIOCHEMICAL REACTIONS (see Table I).

Attempts have been made to subdivide the lacto-bacilli into groups according to their fermentation reactions, *e.g.* the classification of *B. acidophilus* by Rahe (1918). It has not been shown that such a grouping serves any useful purpose or helps in the differentiation of the lacto-bacilli from other organisms, although the fermentation of maltose is held to separate *B. acidophilus* from *B. bulgaricus*. All the strains of the present series were tested for their action on glucose, lactose, saccharose, maltose, mannitol, dulcitol, raffinose, salicin, inulin and litmus milk. Emulsions of a 48 hours' culture of each organism were made in sterile normal saline and the whole culture was distributed in the ten tubes each containing 5 c.c. of 1 per cent. peptone water plus 1 per cent. of the fermentable sugar and Andrade's indicator. Final readings were taken after 10 days' incubation at 37° C. Glucose, maltose and saccharose were almost invariably fermented, lactose less often although the litmus milk was always acidified, which may mean that milk is a better medium for growth than peptone water. Salicin and raffinose were frequently fermented, mannitol and inulin rarely and dulcitol not at all. Glucose, maltose and saccharose were fermented early—usually in 24 hours; acid production in the other fermented sugars was evident in 2–5 days. If the carbohydrate was not fermented there was no apparent growth of the organism. Litmus milk at 37° C. was acidified in 1–2 days and clotted in 3–4 days with decolorisation of the indicator from the bottom of the tube upwards. Strains of Döderlein's bacillus which were not active fermenters when first tested did not acquire any increased fermentative powers when re-tested after six months' subculture. A comparison between the fermentative reactions of Döderlein's bacillus and lacto-bacilli derived from other sources (*B. bifidus*, *B. acidophilus*, Oppler-Boas bacillus and *B. acidophilus odontolyticus*) showed that the strains from carious teeth and from gastric contents (pernicious anaemia, chronic gastritis, cancer of the stomach) are most active in the fermentation of the sugars—in particular they tend to ferment mannitol, which Döderlein's bacillus does not (see Table I).

Table I. *Fermentation-reactions of lacto-bacilli.*

Organism	Glucose	Lactose	Saccharose	Mannitol	Dulcitol	Maltose	Raffinose	Salicin	Inulin	Litmus milk
Döderlein's bacillus, 6 strains	60 (100)	42 (70)	59 (98.3)	4 (6.6)	1 (1.6)	59 (98.3)	47 (78)	44 (73)	3 (5)	A.C.D. 60 (100)
<i>acidophilus</i> (intestine), 3 strains	43 (100)	30 (70)	26 (60.4)	6 (14)	0	43 (100)	—	—	—	A.C.D. 39 A. 4 A.C.D. 9
<i>bifidus</i> (intestine), 3 strains	9	9	8	3	0	9	—	—	—	A.C.D. 9
<i>acidophilus</i> (stomach), 3 strains	6	6	6	6	1	6	3	4	2	A.C.D. 6
<i>acidophilus odontolyticus</i> , 35 strains	35 (100)	35 (100)	31 (88)	34 (97)	13 (37)	32 (95)	23 (67)	35 (100)	27 (80)	A.C.D. 35 (100)

The figures in parentheses denote percentages.

## SEROLOGICAL REACTIONS.

Investigation was directed to finding (*a*) whether there would emerge a serological grouping of Döderlein's bacillus corresponding to the morphological

types, and (b) whether there exists a serological relationship between the vaginal lacto-bacillus and lacto-bacilli derived from other sources.

(a) Agglutinating antisera were prepared against representative strains of each of the morphological types of vaginal origin. Three strains of the most common type and one each of the cocco-bacillus and the strepto-bacillus were used, and antisera with titres of 1 : 640 to 1 : 2560 were obtained after five or six intravenous inoculations at 3-day intervals of emulsions of the living organisms into rabbits. In the performance of the agglutination tests, suspensions of an opacity equal to 500 million organisms per c.c. were prepared from 48-hour serum agar cultures, 0.4 per cent. saline being used throughout in an attempt to prevent a tendency to spontaneous agglutination. However, certain strains, particularly the cocco-bacilli, resisted all attempts to prepare a stable emulsion, *e.g.* growth in broth, repeated washing, vigorous shaking, suspension in 0.001 NaOH (see J. Smith, 1926). The agglutination tubes were incubated at 55° C. for 4 hours and read after standing at room temperature overnight.

*Results.* As has been mentioned, three strains of the commonest type, derived from different individuals, were used to develop antisera. Each of these strains was one of several cultures obtained at different stages of the pregnancy of the respective women. Each antiserum was therefore tested against several strains derived from the same woman, against morphologically similar strains derived from other individuals, and against strains of morphologically different type. The following results were obtained. (1) The same serological type of Döderlein's bacillus tends to persist in the vagina of an individual throughout pregnancy. Thus of four cultures derived from each of two women, all were agglutinated by an antiserum to one of the strains in each case. In the third woman, three out of four cultures were agglutinated. (2) Morphologically similar strains derived from different individuals may or may not be serologically similar. Thus 17 of 20 strains were agglutinated by one of the three antisera (quarter to full titre) whereas only 2 of these strains were agglutinated to eighth-titre by the second antiserum. The third antiserum agglutinated 9 of 12 strains tested against it. Cultures which agglutinated spontaneously are discounted. (3) Cross-agglutination does not occur with strains of morphologically different type. None of the three antisera agglutinated stable strains of the cocco-bacillary or strepto-bacillary varieties, while antisera to the two latter types failed to agglutinate 6 representative strains of the commonest type.

Of 8 cocco-bacillary strains, 5 agglutinated spontaneously: 2 stable strains were not agglutinated by the antiserum (titre 1 : 640) prepared against the remaining strain of the same type. The antiserum to the strepto-bacillus agglutinated one other similar strain, a third was unaffected and the remaining two strains clumped spontaneously in 0.4 per cent. saline.

(b) Regarding the serological identity of Döderlein's bacillus with other lacto-bacilli—*B. acidophilus*, *B. bifidus*, Oppler-Boas bacillus—Jøtten claimed to have proved a close relationship by complement-fixation tests. On the

other hand, Lash and Kaplan failed to find any serological homogeneity in the group of lacto-bacilli by means of agglutination reactions. In the present study, antisera were prepared against *B. acidophilus* (rat intestine), *B. bifidus* and *B. acidophilus odontolyticus*. Ten strains of Döderlein's bacillus, of which two were strepto-bacilli and the others representative of the commonest type, were tested for their agglutinability by each of these three antisera. The chaining cultures were not affected but four of the other strains were agglutinated in low dilutions ( $\frac{1}{16}$ th– $\frac{1}{8}$ th titre) by the antisera to *B. acidophilus* and *B. bifidus*. Conversely, a strain of *B. bifidus* was agglutinated to  $\frac{1}{4}$ th and  $\frac{1}{8}$ th titre respectively by two of the antisera to Döderlein's bacillus, but strains of *B. acidophilus* from stomach and teeth were not agglutinated by the same antisera. These serological relationships among the lacto-bacilli are somewhat similar to those reported by McIntosh *et alii* (1924).

## SUMMARY.

It is concluded from a study of the morphological and cultural characters of 60 strains of Döderlein's vaginal bacillus that the organism belongs to the lacto-bacillus group of bacteria.

Primary culture of Döderlein's bacillus may be readily obtained on lactose blood agar incubated aerobically provided the vaginal flora is of "Grade A" type, but its subsequent propagation on artificial culture media is less likely to be successful.

Three morphological types of Döderlein's bacillus are recognised. Cultural and fermentative tests show that these types are all biologically similar.

There is no serological homogeneity among the strains of Döderlein's bacillus, although there was some evidence of a serological relationship among the members of the commonest morphological type. The same serological type tends to persist in the vagina throughout pregnancy. Döderlein's bacillus and lacto-bacilli derived from other sources are not serologically identical.

## REFERENCES.

- ALBUS, W. R. and HOLM, M. L. (1925). *Proc. Soc. Exp. Biol. and Med.* **22**, 337.  
 CRUICKSHANK, R. and BAIRD, D. (1930). *Edin. Med. J.* **37**, 135 (Proc. of Edin. Obst. Soc.).  
 CRUICKSHANK, J. and CRUICKSHANK, R. (1930). *System of Bacteriology*, **8**. (In press.)  
 GORDON, J. and McLEOD, J. W. (1928). *J. Path. and Bact.* **31**, 185.  
 JÖTTEN, K. W. (1922). *Arch. f. Hyg.* **91**, 143.  
 LASH, A. F. and KAPLAN, B. (1926). *J. Infect. Dis.* **38**, 333.  
 McINTOSH, J., JAMES, W. W. and BARLOW, P. L. (1924). *Brit. J. Exper. Path.* **6**, 175.  
 McLEOD, J. W. and GORDON, J. (1922). *J. Path. and Bact.* **25**, 139.  
 ——— (1923). *Ibid.* **26**, 326.  
 RAHE, A. H. (1918). *J. Bact.* **3**, 420.  
 ROTHER, W. (1922). *Centralbl. f. Bact.* 1. Orig. **88**, 558.  
 SMITH, J. (1926). *J. Hygiene*, **25**, 165.  
 THOMAS, S. (1928). *J. Infect. Dis.* **43**, 218.



# SPECIFIC AND NON-SPECIFIC SERUM REACTIONS IN TYPHUS FEVER.

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## INTRODUCTION.

In a previous paper (Felix and Rhodes, 1931) the occurrence of agglutinins for different serological types of *B. proteus* X in the serum of patients suffering from various forms of typhus and typhus-like diseases was discussed and illustrative data presented. The conclusion arrived at was that these diseases represent serological varieties of typhus and that antigenically their respective viruses correspond to serological types of *B. proteus* X.

In the present paper the nature of this agglutination reaction is discussed.

It is well known that in the past this problem was the subject of much controversy. On the one hand the X 19 reaction in typhus fever has been generally recognised as one of perfect "clinical" specificity and is now accepted as one of the most reliable serological tests used in routine diagnosis. On the other hand, the views held with regard to the nature of the reaction are still as controversial as they were ten years ago: in the opinion of most bacteriologists—at least as far as it is expressed in text-books—the reaction is non-specific in nature, unrelated directly with the typhus virus, while those who hold the opposite view represent a very small minority.

Numerous theories have been put forward with the view of proving the

hypothesis of the non-specificity of the Weil-Felix reaction (for references see Wolff, 1922; Wilson, 1929; Otto and Munter, 1930). The most favoured amongst them are (1) the paragglutination theory (Otto, 1919), (2) the theory which postulates peculiar and constant physico-chemical changes of the blood-serum of typhus patients (Epstein, 1919, and others) and (3) Wilson's (1920, 1927) hypothesis according to which the X 19 reaction is one instance of "heterologous" agglutination of which other instances had been described by Wilson (1909, 1910) and others. It is not proposed to consider here these theories nor several others which have been suggested by other workers. In this paper one argument only will be discussed which has been most widely used in the course of this controversy and has served as the common basis of all the theories mentioned. It is the alleged fact that it is a peculiarity of the blood serum of typhus patients to develop agglutinins for a variety of bacteria and that *B. proteus* X 19 is only one out of a number of organisms which can be used for the detection of these agglutinins. On the Continent the conception has been formulated of a "polyagglutinating" capacity peculiar to the blood serum of typhus patients, while in this country the phenomenon is usually ascribed to the presence of heterologous agglutinins, so-called.

## I. REVIEW OF EARLIER LITERATURE.

### (a) "*Heterologous*" agglutinins.

The occurrence of "heterologous" agglutinins in the serum of patients and of immunised animals has been recorded since the early days of the study of the agglutination phenomenon (for references see Paltauf, 1904, 1913; Wilson, 1909, 1920). The term was introduced to separate the so-called group agglutination, which is due to common group antigens, from an agglutination reaction occurring between a heterologous serum and an organism which does not possess any demonstrable group antigen in common with the organism homologous to the serum. The differentiation between group agglutinins and heterologous agglutinins has been based on the result of the absorption test; the former are removed from the serum by absorption with the homologous organism while the latter are left intact.

Many of the earlier observations on heterologous agglutinins, especially those recorded in the case of human patients' serum, are probably due to an erroneous interpretation of results, caused by the imperfect knowledge of various sources of error which have been elucidated by subsequent investigations. Some of these pitfalls of the agglutination reaction are: the incidence in the normal serum of man and animals of agglutinins often in high titre, especially for *B. coli* and other intestinal bacteria; re-stimulation of persisting agglutinins, due to a previous infection or inoculation, under the non-specific stimulus of another infection or immunisation; non-specific serum agglutination of suspensions of partially "rough" organisms; various degrees of community of antigen amongst members of different species. However, other

observations clearly indicate that instances of significant rise and decline in the titre of heterologous agglutinins have been established beyond doubt.

Heterologous agglutinins in the serum of immunised animals have been demonstrated first by Posselt and Sagasser (1903) and also by Ballner and Sagasser (1904). In human patients' serum they have been carefully studied by Wilson (1909, 1910) in cases of cerebro-spinal fever and typhus fever. *B. coli* and *B. aquatilis alcaligenes*, an organism isolated from Belfast tap water and resembling the *B. faecalis alcaligenes*, were found to react significantly with the serum of patients suffering from cerebro-spinal fever. In one case the curves of agglutinin formation for the latter organism and for the meningococcus were compared thoroughly and were found to a certain extent parallel. The blood serum of typhus patients agglutinated several organisms isolated from these cases, viz. diplococci from the blood, *B. coli communis* from the urine and a variant of *B. coli communis* from the faeces, designated Bacillus U. From these observations Wilson reached the conclusion that heterologous agglutinins for various saprophytic organisms occur in the patients' serum in the course of these two diseases.

The origin of heterologous agglutinins and their relationship to the normal agglutinins have not yet been explained satisfactorily. It is not yet decided whether the rise and decline of heterologous agglutinins are due to the antigenic action of the infecting organism or to that of the saprophytic organism concerned, which is harboured by the particular individual and favoured by the conditions created by an intercurrent infection or immunisation. Another theory is that heterologous agglutinins are identical with normal agglutinins increased by a non-specific stimulus; and a further theory explains the phenomenon by assuming physical and chemical changes in the blood serum, altered "globulins" being held to be the responsible factors.

(b) *The "polyagglutinating" capacity of typhus serum.*

There is no doubt that the serum of some typhus patients, like some other sera, does agglutinate various bacteria in higher dilution than normal serum.

The term "polyagglutinating" capacity of typhus serum was introduced by Weltmann (1916) to denote the supposed peculiarity possessed by the serum of typhus patients of agglutinating various kinds of bacteria. This view had been formed because each of the numerous workers, who at some time or other had described a presumed causal organism of typhus fever, had also described an agglutination reaction in the attempt to prove its etiological significance. These results, however, could not be confirmed and the analysis of the data referring to these agglutination tests clearly indicated that they had been obtained by inaccurate methods (for references see Felix, 1918; Weil, 1920). Indeed, some of the authors themselves stated that their results were not satisfactory and could not carry weight. Nevertheless, undue importance was attached to their observations by subsequent workers (Rocha-Lima, 1919, and others) and the fact that for many years they were almost

generally accepted as valid evidence of the polyagglutinating property peculiar to typhus serum had a decisive effect on subsequent research on typhus fever. In a recent paper Weigl (1930) admitted this misleading effect on his own previous work (Weigl, 1923, 1924) and justly stated that nothing but a bad reputation had been preserved with regard to the numerous organisms which in the past had been brought into etiological relationship with typhus, mainly or partly on the basis of inadequate serological tests.

In addition to various organisms isolated from cases of typhus fever others have been described which are not related in such a way to the disease, but are still considered to be specifically agglutinated by the patients' serum. *Br. melitensis* may serve to illustrate how imperfect technique and imperfect judgment have combined in the formation of the opinion that a polyagglutinating capacity is peculiar to typhus serum and responsible also for the X 19 reaction. Nicolle and Comte (1910) published the following results of agglutination tests with *Br. melitensis* and the serum of typhus patients in Tunis (see Table I).

Table I. *Agglutination of Br. melitensis by the serum of sixty-eight typhus patients in Tunis (Nicolle and Comte, 1910).*

Titre of agglutination	No. of cases	%
1 : 10	12	17.64
1 : 20 till 1 : 50	18	26.47
1 : 50	14	22.5
1 : 100	1	
Total of agglutinating sera	45	66.61

They found that in 66 per cent. of cases an agglutinating action of the serum on *Br. melitensis* had been established, although they pointed out at the same time that in undulant fever a titre lower than 1 : 50 should not be taken as diagnostic of the disease. They recommended an agglutination test with *Br. melitensis* for routine diagnosis of typhus cases. Although the presumed peculiar action of typhus serum on *Br. melitensis* could not be confirmed by other workers (Negre and Raynaud, 1911; Rizutti and Scordo, 1912; Markl, 1913; Felix, 1918), and although it is known that in the blood of normal persons agglutinins may be present for this organism, sometimes up to a titre of 1 : 50 or 1 : 100, nevertheless the statement was repeated again and again in the literature on this subject that, according to Nicolle and Comte, typhus serum agglutinates *Br. melitensis* in 66 per cent. of cases (Rocha-Lima, 1919; Wolff, 1922; Otto and Munter, 1930, and others).

The non-specific re-stimulation by typhus fever of agglutinins for *B. typhosus* due to inoculation or previous enteric infection (Weil and Felix, 1916; Felix, 1917, 1929) was also considered, but mistakenly, to be peculiar to typhus serum.

In a previous paper (Felix, 1918) the "polyagglutinating" action of typhus and non-typhus sera on a number of "polyagglutinable" organisms, which, it was suggested, were specifically agglutinated by typhus serum, was compared

with the action of the same sera on *B. proteus* X 19. The non-specificity of the former was as evident as was the specificity of the latter reaction, since the polyagglutinable organisms reacted in a similar manner with both the typhus and the non-typhus sera while the X 19 did not. It was demonstrated in that paper and again emphasised by Weil (1920) that in comparative investigations of that kind it was indispensable to pay attention to the proper selection of adequate controls. It is obvious that in order to prove a supposed peculiar relationship between typhus serum and a polyagglutinable organism the latter ought also to be tested with sera from such other pathological conditions as are also known to cause an increased agglutinating power for various saprophytic organisms. Tuberculosis, syphilis, leprosy and other severe diseases are known to belong to this category. If, however, the controls are taken indiscriminately from cases of various diseases, including mild pathological conditions, surgical cases or even healthy individuals, then the polyagglutinating capacity developed in the course of typhus and other severe diseases may simulate specificity.

This essential condition of experiment was entirely neglected in the work concerning *B. pyocyaneus* Z 1 (Kreuscher, 1918; Neukirch and Kreuscher, 1919) and *B. agglutinabilis* U<sub>2</sub> (Wilson, 1927), the two organisms which have received much prominence in typhus literature. Wilson (1929) still holds the view that for the serological diagnosis of typhus fever these organisms "are often found almost equally serviceable" (p. 319) with the *B. proteus* X strains. Otto and Munter (1930) make the equally unwarranted statement that these strains of *B. pyocyaneus* and *B. coli* exhibit the same "outspoken specific affinity to the typhus patients' serum" (p. 1167) as *B. proteus* X strains do. With regard to *B. pyocyaneus* Z 1 the statements of the authors to whom they refer read as follows: "It has to be decided by further investigations and controls how far the phenomenon is strictly specific" (Kreuscher, 1918). And later: "It has been shown that the relationship between typhus sera and Z 1, which does not deserve the name specificity, is at least a peculiarity which might be taken into consideration in connection with the question as to the nature of the Weil-Felix reaction" (Neukirch and Kreuscher, 1919, p. 82).

It has been shown by Weil and Felix (1921) that rabbits infected with typhus virus invariably develop agglutinins for *B. proteus* X 19 but none for *B. pyocyaneus* Z 1 nor for other polyagglutinable organisms. The non-specificity of the agglutination reaction which Z 1 gives with the serum of some typhus patients has, therefore, been proved experimentally. Since *B. agglutinabilis* U<sub>2</sub> has not hitherto been tested in similar experiments and in view of the misleading statements previously mentioned a comparative investigation of the reactions which U<sub>2</sub> and X 19 give with human patients' sera was undertaken (see below).

(c) *Weltmann's globulin test and the Wassermann reaction in typhus serum.*

Weltmann (1916, 1917) described the phenomenon that in typhus the dilution (1 : 10) of the patients' serum with distilled water produces turbidity and suggested the use of this reaction for the diagnosis of typhus cases. The method was soon shown to be worthless since the test was not uniformly positive in typhus and was also met with in various other conditions. A similar phenomenon was known to occur with lues serum (Klausner, 1908) and is due, in both instances, to an alteration of the serum globulins (Epstein, 1919).

A transient positive Wassermann reaction in typhus was first described by Delta (1915), Papamarku (1915), Gotschlich, Schürmann and Bloch (1915) and later confirmed by Bauer (1921), Cruickshank (1927) and others. It does undoubtedly appear in a great proportion of cases in the course of the disease and becomes negative again during convalescence. It is known that the same phenomenon occurs in various infectious diseases, other than syphilis, such as malaria, leprosy, etc.

Both these non-specific serum reactions, though evidently not peculiar to typhus serum, were also brought into connection with the Weil-Felix reaction and it was suggested that all of them were due to one common factor—viz. to changes in the physico-chemical state of the serum. It is obvious that the supporters of this view entirely neglect the fact, established by those who have had the largest experience with typhus in various parts of the world, that non-specific reactions with X 19 do not occur in all those diseases which are known to produce a positive Wassermann reaction or an increase in the globulins of the blood serum. In typhus the independence of the result of the Wassermann reaction and of agglutination and complement fixation with X 19 has been shown by Reichenstein (1917) and the lack of correlation between the alteration of serum globulins and the titre of agglutination with X 19 is evident even from the data published by Epstein and Morawetz (1917).

## II. COMPARATIVE OBSERVATIONS ON SERUM AGGLUTINATION WITH *B. PROTEUS* X 19 AND *B. AGGLUTINABILIS* U<sub>2</sub> (WILSON).

(a) *Agglutination with normal and immune rabbit serum.*

Prof. W. J. Wilson, Belfast, very kindly sent me a culture of his coliform strain *B. agglutinabilis* U<sub>2</sub>. Its cultural characters were described by Wilson (1927) and in my hands it differed from the original description in one respect only. Wilson stated that in young cultures the bacilli were actively motile, while in my experience over a period of three years they were non-motile when grown on ordinary agar. Fletcher, Lesslar and Lewthwaite (1929) also described it as non-motile. Accordingly, no H agglutination has been observed with U<sub>2</sub>, either in normal and immune sera from animals or in human sera. The agglutination reactions obtained with this organism and recorded in this paper were exclusively of the O type and they were compared only with the

O agglutination of X 19. It is known that only this type of agglutination is the decisive criterion in the diagnosis of typhus fever; further, it is established that normal agglutinins in human serum and in that of the customary small laboratory animals are O agglutinins (Rotky, 1921; Schiff, 1922). For these reasons no attention was paid throughout this investigation to H agglutinins for *B. proteus* X 19 which exceptionally are met with in human serum and still more rarely in small laboratory animals.

Since "roughness" of bacterial suspensions is one of the most important sources of error in agglutination tests much attention was directed to this point, and according to Arkwright's (1921) technique controls with saline solutions of increasing salt concentration (up to 6.8 per cent. NaCl) were included in each agglutination test. Both strains,  $U_2$  and X 19, invariably gave stable suspensions indicating perfect "smoothness."

Wilson (1927) found in absorption tests with typhus serum that the agglutinins for  $U_2$  and X 19 were separate and distinct. Nevertheless, it was carefully tested whether the two organisms do not share a common O antigen. Serum from twenty-five rabbits immunised with genuine and with variant strains of *B. proteus* X of all the known types (see Felix and Rhodes, 1931) and serum from five rabbits immunised with *B. agglutinabilis*  $U_2$  were tested for cross-agglutination. In accordance with Wilson's finding no community of antigen could be established between the two organisms, although the sera used reacted up to high titres with their respective homologous organisms.

These tests, however, revealed the interesting fact that the serum of normal rabbits contains agglutinins for  $U_2$  in much higher titre than for X 19. It has been shown by Weil and Felix (1921) that normal rabbit serum in dilution 1 : 10 very rarely reacts with either the H or O variants of X 19 and that agglutinins often cannot be detected even in a dilution 1 : 5.

Table II. *Agglutination of B. agglutinabilis*  $U_2$  and *B. proteus* X 19 by thirty-two sera of normal rabbits.

Titre	No. of sera reacting with	
	<i>B. agglutinabilis</i> $U_2$	<i>B. proteus</i> X 19
< 1 : 10	1	29
1 : 10	8	3
1 : 20	17	0
1 : 50	4	0
1 : 100	2	0
1 : 200	0	0
Total	32	32

In Table II the titre of normal agglutinins occurring in rabbit serum for *B. agglutinabilis*  $U_2$  and *B. proteus* X 19 is shown. The difference is very striking.

(b) *Agglutination with normal and immune chicken serum.*

Friedberger, Zorn and Meissner (1922), using immune sera from chickens, succeeded in demonstrating community in the O antigen of the X strains and

of *B. pyocyaneus* Z 1, the polyagglutinable organism previously mentioned. Four chickens were, therefore, immunised with the *Proteus* strains X 19, X<sub>2</sub>, XK and a variant derived from X 19, respectively, and one chicken was immunised against *B. agglutinabilis* U<sub>2</sub>. Sera with high O titres (up to 1:10,000) for the homologous organisms were obtained. No trace of overlapping agglutinins could, however, be disclosed between U<sub>2</sub> and the four strains of *B. proteus* X.

Again, the titration of the sera of the chickens, before immunisation was begun, showed a very great difference in the amount of normal agglutinins for U<sub>2</sub> and X 19.

Table III. *Agglutination with normal chicken serum.*

Chicken no.	Serum dilution	Agglutination with strains		
		<i>B. proteus</i> X 19		<i>B. agglutinabilis</i> U <sub>2</sub>
		H variant	O variant	
1	1:25	+	+ ±	++
	1:50	-	±	+ ±
	1:100	-	-	±
	1:200	-	-	-
2	1:25	+	++	+++
	1:50	-	+	+++
	1:100	-	-	+
	1:200	-	-	-
3	1:25	+	++	+++
	1:50	-	±	+++
	1:100	-	-	+ ± ±
	1:200	-	-	+
4	1:500	-	-	-
	1:25	±	+ ±	+++
	1:50	-	±	+++
	1:100	-	-	+++
5	1:200	-	-	++
	1:500	-	-	±
	1:25	++	++ ±	+++
	1:50	±	+	+++
Controls NaCl (%)	1:100	-	±	+ ± ±
	1:200	-	-	+
	1:500	-	-	-
	0, 85	-	-	-
	1, 7	-	-	-
	3, 4	-	-	-
	6, 8	-	-	-

Agglutination with fresh saline suspensions of living bacteria. Total volume 1 c.c.

Reading after 20 hours; 2 hours' incubation at 37° C., then at room temperature.

+++ , ++ , + , ± = varying degrees of agglutination, estimated with the naked eye.

The degree of agglutination indicating the "titre" in all the other tables corresponds to the sign ±.

In Table III the results obtained with U<sub>2</sub> and with both the H and the O variant of X 19 are reproduced. That O agglutination with the O culture generally gives a higher titre than with the H culture has been shown by Weil and Felix (1917, 1921), the proportion in the titres reached being about 2:1. In the following experiments, however, the H culture only has been used, since almost all the data published on the Weil-Felix reaction have been obtained with the H variant of X 19.

The difference in the titre of normal agglutinins for *B. agglutinabilis* U<sub>2</sub> and for X 19 was as conspicuous with chicken sera as with rabbit sera.



*(c) Agglutination with normal guinea-pigs' serum.*

The guinea-pig is known for its poor capacity for producing immune bodies and most of the earlier workers also agree that guinea-pigs' serum is remarkably poor in normal agglutinins for various organisms. It is the animal placed at the bottom of the scale in which various animal species have been arranged according to the amount of normal agglutinins contained in their blood serum (Bürge, 1907; Mamlok, 1909).

The very great difference in the titre of normal agglutination with  $U_2$  and X 19, previously established with rabbit and chicken sera, was also found with the serum of normal guinea-pigs (see Table IV).

Table IV. *Agglutination of B. agglutinabilis  $U_2$  and B. proteus X 19 by thirty sera of normal guinea-pigs.*

Titre	No. of sera reacting with	
	$U_2$	HX 19
<1 : 10	8	28
1 : 10	15	2
1 : 20	6	0
1 : 50	0	0
1 : 100	1	0
1 : 200	0	0
Total	30	30

*(d) Agglutination with normal human sera.*

Sera from out-patients which had been tested for the Wassermann reaction and had been found to give a negative test were used as "normal" human sera. Seventy-two samples of such serum were kindly supplied by Dr P. Fildes, London Hospital, and by Dr A. B. Rosher, Charing Cross Hospital. Only samples of serum were employed for agglutination tests which had not been inactivated. This precaution was taken because O agglutinins, to which type human normal agglutinins belong, generally possess remarkably low resistance to heat (Weil and Felix, 1917; Felix and Olitzki, 1929). Eight or ten days usually elapsed between the date of collecting the sera and that of testing them for normal agglutinins.

Simultaneously with the agglutination test Weltmann's globulin test was also performed with each serum. Two sets of serum dilutions 1 : 10 were put up in parallel, with normal saline and with distilled water, respectively, and were allowed to stand for 15 minutes at room temperature. Distinct turbidity occurring in the serum dilution with distilled water was then taken as indicating a positive reaction. None of the Wassermann negative sera gave a positive globulin test.

From Table V it is seen that in human serum, too, the titre of normal agglutinins for *B. agglutinabilis*  $U_2$  exceeds that for *B. proteus* X 19 to a similar extent to that previously shown in various animal species. When the figures tabulated in Table V are compared with those specified in the previous tables

it will be found that, with regard to the amount of normal agglutinins contained in the blood serum, man holds a position intermediate between the guinea-pig

Table V. *Agglutination with normal human sera.*  
(*Seventy-two Wassermann negative sera.*)

Titre	No. of sera reacting with	
	U <sub>2</sub>	HX 19
<1 : 50	19	65
1 : 50	28	7
1 : 100	21	0
1 : 200	4	0
1 : 500	0	0
Total	72	72

and the rabbit on the one hand and the chicken on the other hand. This result is in keeping with the older work of Bürgi (1907) and others.

(e) *Agglutination with Wassermann positive sera from patients suffering from syphilis and leprosy.*

One hundred and thirty-eight samples of Wassermann positive lues serum were obtained from the two sources which supplied the Wassermann negative sera recorded in Table V. In both series the sera were manipulated in exactly the same way and in each particular experiment positive and negative sera were tested against the same bacterial suspensions side by side. The constancy of the sensitiveness to O agglutinins of both strains used was regularly controlled by standard rabbit immune sera and as an additional control positive and negative sera were repeatedly re-tested after having been stored in the ice box. The accurately quantitative character of the method in use was thus guaranteed throughout the long course of this investigation.

All the Wassermann positive sera were also tested by Weltmann's globulin test with the result that a positive reaction was obtained in a considerable proportion of cases. The degree of turbidity observed in the globulin test often corresponded to the intensity of the Wassermann reaction, a result which had been recorded by Dr Rosher in the case of fifty-seven sera supplied by him; however, exceptions to this rule were also noted, a fact well known from the observations made by earlier workers.

Table VI. *Agglutination with 138 Wassermann positive lues sera.*

Titre	No. of sera reacting with	
	U <sub>2</sub>	HX 19
<1 : 50	30	126
1 : 50	38	12
1 : 100	41	0
1 : 200	21	0
1 : 500	6	0
1 : 750	1	0
1 : 1000	1	0
1 : 2000	0	0
Total	138	138

High titres of agglutination with  $U_2$  were observed in numerous cases with these positive lues sera while in no instance did the titre for X 19 exceed that previously established with normal human serum. It is not proposed to compare the results obtained with normal and lues sera in terms of percentage, because of the comparatively small totals in both series. The figures in Tables V and VI clearly demonstrate that the agglutinating property of Wassermann positive lues sera is considerably increased for *B. agglutinabilis*  $U_2$  while it does not exceed that of normal human serum with regard to *B. proteus* X 19. No constant relationship was, however, established between the intensity of the globulin and the Wassermann reactions on the one hand and the titre of agglutination with  $U_2$  on the other hand.

Analogous results were obtained with the serum from patients suffering from leprosy (see Table VII). I owe these sera to the kind co-operation of Dr G. Stuart, Department of Health, Jerusalem, who also tested them for the Wassermann reaction and supplied the clinical data recorded in Table VII. The blood was drawn from 14 leper cases in Jerusalem on 22. ix. 30 and the sera, which had been neither inactivated by heat nor preserved by any disinfectant, were tested for their agglutinin content and by Weltmann's globulin test on 16. x. 30 in London. For reasons to be explained later the *Proteus* strains  $X_2$  and XK and the *B. pyocyaneus* Z 1 have also been included in this experiment which is reproduced in detail.

Table VII. *Agglutination test with fourteen leper sera.*

Patient no.	Type of disease	Wassermann reaction			Titre of agglutination with strains				
		Citron's evaluation	M.R.C. deviation in M.H.D.	Weltmann's globulin test	$U_2$	HX 19	HX 2	HXX	Z 1
1	Nodular	++	3	±	200	0	0	0	100
2	Mixed	++++	5	+	100	50	0	0	50
3	Mixed	++	3	-	100	0	0	0	50
4	Nervous	-	0	±	50	0	0	0	200
5	Nodular	++++	5	±	200	0	50	50	100
6	Nodular	++++	5	±	0	0	0	0	200
7	Mixed	-	0	-	50	50	0	0	100
8	Nodular	-	0	-	100	0	0	0	0
9	Nodular	++++	5	+++	100	0	50	0	200
10	Nodular	++++	5	±	100	0	0	0	50
11	Nervous	-	0	-	50	0	0	0	50
12	Mixed	++	3	+	500	0	0	50	100
13	Mixed	-	0	-	50	0	0	0	50
14	Mixed	++++	5	+	200	0	0	0	50

Titre 0 = a negative result in dilution 1 : 50.

A more detailed analysis of the figures in Table VII is preferably reserved for a subsequent section of this paper. For the present two facts only may be emphasised: (i)  $U_2$  is agglutinated distinctly higher by leper sera than by normal human sera (see Table V), while in the case of X 19 no difference whatever is detectable; (ii) high titres for  $U_2$  are met with only in sera recorded as positive in both the Wassermann and the Weltmann tests (cases Nos. 1, 5,

12, 14); however, a strongly positive Wassermann or Weltmann reaction does not necessarily coincide with a high titre for  $U_2$  (cases Nos. 2, 6, 9).

(f) *Agglutination with sera from patients suffering from European classical typhus and from various typhus-like diseases.*

In Table VIII are recorded the titres for  $U_2$  and X 19 obtained with the serum from cases of classical European typhus and of those typhus-like diseases occurring in various parts of the world which, though not louse-borne, belong to the same serological variety, *i.e.* react with type X 19 of *B. proteus* X. The action of these sera on various types of *B. proteus* X has been described in a previous paper (Felix and Rhodes, 1931), where they have been specified in Tables I and VII under the numbers corresponding to those in Table VIII of the present paper. The American cases only, Nos. 8-17, had to be omitted in the previous paper, since their sera were preserved with glycerole which was found to cause non-specific agglutination of suspensions of the two strains  $HX_2$  and  $OX_2$ . On *B. agglutinabilis*  $U_2$  and on X 19 glycerole has no agglutinating effect; the glycerole sera could, therefore, be included in Table VIII.

As has been stated in the paper mentioned above rather long intervals usually elapsed between the date of collecting these exotic sera and that of testing them for their agglutinin content. This, however, does not vitiate the conclusions drawn from the examination of these sera, since the agglutinins for  $U_2$  and X 19 considered here belong to the same type (O) and equally withstand the effect of ageing, whether the serum is preserved with or without the addition of glycerole (Wilson, 1927). None of the sera could, however, be tested for the Wassermann and the Weltmann reaction, for which purposes old sera are not suitable.

In Table VIII, as in all the tables in this paper, the degree of agglutination indicating the maximum titres corresponds to the sign  $\pm$ , estimated with the naked eye, while in the previous paper (Felix and Rhodes, 1931) the sign + has been taken to mark the titre. This was necessary in order to increase the fineness of reading the low titre reactions between X 19 and normal agglutinins, which otherwise could not have been compared adequately with the much higher titres for  $U_2$ . Consequently, in Table V the figure stated for X 19 with normal agglutinins in human serum is higher by one serum dilution than that usually quoted, namely 1 : 50 instead of 1 : 25, and a similar difference is seen when the X 19 titres of the typhus sera in Table VIII are compared with the corresponding figures in the previous paper.

In Table VIII the titre O denotes a negative result in the dilution 1 : 200. Reactions with stronger serum dilutions had to be disregarded owing to the fact that they often occur with normal human serum (see Table V). According to the accepted rule even the titre of 1 : 200 ought to be considered as insignificant, since it is met with in four out of seventy-two normal sera (Table V).

From comparison of Tables V-VIII it becomes perfectly clear that the

positions held by the two organisms under examination differ profoundly from each other. The agglutination of *B. agglutinabilis* U<sub>2</sub>, occurring with relatively high titres in normal human serum, is distinctly higher with the serum from patients suffering from syphilis or leprosy and still more so with typhus serum; it is, however, far from being a constant feature of typhus serum. On the other hand, the agglutination of *B. proteus* X 19 with its very much lower incidence and titres in normal individuals is not in the slightest degree affected by the syphilitic or leprosy infection, but it is invariably very much enhanced by a typhus infection.

So far as *B. proteus* X 19 is concerned Tables V–VIII once more demonstrate that its reaction with typhus serum is, in the strict meaning of the word, “peculiar” to the typhus infection. With regard to *B. agglutinabilis* U<sub>2</sub>, however, these tables clearly show, that its reaction with typhus sera is not

Table VIII. *Agglutination of B. agglutinabilis U<sub>2</sub> and B. proteus X 19 by sera from cases of typhus and typhus-like diseases.*

Typhus patients from United States of America	Case no.	Titre of agglutination with strains		Typhus patients from	Case no.	Titre of agglutination with strains	
		U <sub>2</sub>	HX 19			U <sub>2</sub>	HX 19
	1	0	500	Poland	1	500	5000
	2	0	1000		2	200	5000
	3	0	500		3	200	500
	4	1000	5000		4	500	10000
	5	0	2000		5	200	5000
	6	0	1000		6	200	500
	7	2000	5000		7	0	1000
	8	0	5000		8	0	2000
	9	0	200		9	200	2000
	10	1000	5000		10	500	5000
	11	200	1000	Ireland	1	2000	5000
	12	0	500	Australia	1	0	20000
	13	0	2000	Malaya	1	1000	5000
	14	2000	2000	(Group “W”)	2	1000	2000
	15	200	5000		3	500	2000
	16	200	1000		4	0	5000
	17	0	2000				

Titre 0 = a negative result in dilution 1 : 200.

“peculiar” to this disease, since similar though somewhat lower reactions occur in cases of syphilis and leprosy.

It has been already mentioned that neither the Wassermann nor the Weltmann reaction could be attempted with the typhus sera, the samples having been either too old or preserved with glycerole. It has, therefore, not been established whether or not these two reactions were positive in those typhus sera which strongly agglutinated the *B. agglutinabilis* U<sub>2</sub>. It seems probable that this coincidence obtains in typhus as well as in leprosy and syphilis. If this assumption were shown to be correct it would also offer an explanation of the fact that the incidence of high titre agglutinations with U<sub>2</sub> is markedly higher in typhus than in syphilis or leprosy. It has been emphasised by Weltmann (1916, 1917) and by Epstein (1919) that the alterations of serum globulins occurring as a result of syphilis and of typhus infection are

not identical. This conclusion was drawn from the fact that in the two conditions the globulin test requires different dilutions of serum with distilled water and different times of observation in order to yield optimum results. According to these workers it would appear that the increase in water-insoluble globulin is greater in typhus than in lues serum and this difference may account for the somewhat stronger agglutinating effect on  $U_2$  by typhus sera.

An attempt was made to analyse the mechanism of the non-specific, heterologous agglutination of "polyagglutinable" organisms like  $U_2$  by "polyagglutinating" sera such as are exemplified by syphilis, leper and typhus sera. This has no direct bearing on the typhus reaction with X 19, since this organism is not "polyagglutinable" and its specific relationship to the typhus virus has been established experimentally (Weil and Felix, 1921, and others). The following experiments might, however, contribute to the still imperfect knowledge of "heterologous" agglutination.

### III. NON-SPECIFIC AGGLUTINATION BY GELATINE.

An interesting hypothesis was suggested long ago by Bürgi (1907) with the view of reconciling some apparently inconsistent facts established in the study of normal agglutination. On the one hand, data are available (see Gibson, 1930) showing that agglutinins for various organisms, simultaneously present in a normal serum, are capable of specific absorption in exactly the same way as are immune agglutinins due to specific sensitisation. On the other hand, it has been established by numerous workers that the content of normal agglutinins varies considerably with different animal species and that their normal sera are either generally rich or generally poor in agglutinins for various kinds of bacteria. The latter fact was considered by Bürgi (1907) as highly suggestive of a non-specific factor being involved in the phenomenon. His theory, calculated to reconcile the specific and non-specific features of normal agglutination, assumes a combined action of two factors, analogous to the two stages of the agglutination phenomenon, viz. specific sensitisation by the agglutinin and non-specific flocculation by electrolytes. Accordingly, specific agglutinins for various kinds of bacteria, present in small amounts in normal sera, are held responsible for the first stage of the phenomenon, the titre of the reaction, however, being determined by the non-specific effect of colloids present in the serum of different animals in varying amounts and in different physico-chemical conditions.

Now, marked physico-chemical changes in the serum proteins have been established as a result of pathological conditions like typhus, syphilis, leprosy, which give rise to the development in the patient's serum of polyagglutinating properties. It seemed, therefore, advisable to analyse the latter phenomenon on the lines of Bürgi's theory.

Gelatine was selected as the colloid to be tested since there exists no method of isolating serum globulins free from serum agglutinins. Moreover, the

agglutinating effect of gelatine on bacterial suspensions had already been studied by Weil (1904), who concluded that the mechanism of this non-specific agglutination tallies with that of specific serum agglutination.

Table IX. *Agglutination by gelatine.*

% gelatine in normal saline	Agglutination with strains								
	<i>B. proteus</i> X 19			<i>B. proteus</i> XK			<i>B. typhosus</i> No. 901		
	H	O		H	O		H	O	<i>B. paratyphosus</i> B
	variant	variant		variant	variant		variant	variant	
3, 0	+H	-		++H	(±) O	+O	++H+O	±O	++H+O
1, 0	-	-		(±)	-	-	+±H	(±)	(±) H
0, 5	-	-		-	-	-	-	-	-
0, 1	-	-		-	-	-	-	-	-

3 per cent. gelatine in normal saline, adjusted to pH 7.4, sterilised at 100° C., prepared in bulk and further dilutions made with saline.

Fresh saline suspensions of living bacteria. Total volume 1 c.c.

Reading after 20 hours' incubation at 50° C.

± = last degree of agglutination estimated with the naked eye.

(±) = traces, estimated by means of a magnifying lens.

In Table IX the two polyagglutinable organisms *U*<sub>2</sub> and *Z* 1 are compared with H and O variants of *B. proteus* X and of some *Salmonella* types with regard to their sensitiveness to the action of gelatine. It is interesting to note that the colloid acts on both the H and the O antigenic components, leading to typical floccular and granular agglutination. As in specific serum agglutination so here also the titre of H is higher than that of O agglutination. The sensitiveness of the O reaction with the polyagglutinable strains *U*<sub>2</sub> and *Z* 1 is, however, not much more marked than that with the other organisms, which do not belong to this category.

Weil (1904) also described a summation effect of the action of gelatine and agglutinating serum. Minute amounts of both substances, incapable of causing visible agglutination when acting separately on the bacterial suspension, produced a distinct effect by combined action. Bacteria which were and were not polyagglutinable were, therefore, subjected to similar tests (Table X).

Table X. *Combined action of gelatine and specific immune agglutinins.*

Agglutination of homologous rabbit O immune sera with strains											
<i>B. proteus</i> OX 19			<i>B. typhosus</i> O 901			<i>B. agglutinalibis</i> <i>U</i> <sub>2</sub>			<i>B. pyocyaneus</i> <i>Z</i> 1		
Serum dilution	In saline	In saline +1 % gelatine	Serum dilution	In saline	In saline +0.5 % gelatine	Serum dilution	In saline	In saline +1 % gelatine	Serum dilution	In saline	In saline +1 % gelatine
1:5000	+	++±	1:20000	(±)	±	1:5000	+±	+++	1:2000	+	+++
1:10000	(±)	+±	1:50000	-	±	1:10000	±	++	1:5000	±	++
1:20000	-	+	1:75000	-	(±)	1:20000	-	+	1:10000	-	+
1:50000	-	-	1:100000	-	-	1:50000	-	(±)	1:20000	-	±
									1:50000	-	(±)
Controls	-	-	Controls	-	-	Controls	-	-	Controls	-	(±)

Technique, see Table IX.

It is seen from Table X that the summation effect described by Weil was produced in every instance. No difference between the various organisms could, however, be established as regards their sensitiveness to the combined specific and non-specific action.

IV. NON-SPECIFIC STIMULATION OF AGGLUTINATING CAPACITY  
BY HETEROLOGOUS IMMUNISATION.

Although the experiments with gelatine had failed to reveal any marked difference in sensitiveness to non-specific agglutination between polyagglutinable and non-polyagglutinable bacteria, it was thought possible that other colloids, like serum proteins, might in this respect act differently. Serum from horses undergoing hyper-immunisation with unrelated bacteria (certain anaerobes) was, therefore, tested for heterologous agglutinins, since changes in the quantity and quality of serum globulins are well known in that condition.

Table XI. *Agglutination with serum of horses before and after immunisation with various types of anaerobes.*

No.	Horse serum	Homo- logous to serum	Titre of agglutination with strains							
			<i>B. proteus</i> X 19		<i>B. proteus</i> XK		<i>B. agglu- tinabilis</i>	<i>B. typhosus</i> No. 901		No. 901
			H variant	O variant	H variant	O variant	U <sub>2</sub>	H variant	O variant	
1	Before immunisation	0	0	100	0	100	500	100	200	
	After immunisation with <i>B. tetani</i>	500	0	100	0	100	2000	100	200	
2	Before immunisation	0	0	100	100	200	100	100	200	
	After immunisation with <i>Vibrio septique</i>	500	0	100	100	200	500	100	200	
3	Before immunisation	0	100	200	100	200	500	200	500	
	After immunisation with <i>B. welchii</i>	1000	100	200	100	200	2000	200	500	

Titre 0 = a negative result in dilution 1 : 100.

Horses immunised by intravenous injections of washed bacilli heated to 100° C. for two hours.

As is seen from Table XI, the polyagglutinable strain U<sub>2</sub> differs markedly from the other organisms tested at the same time, since it is the only one agglutinated by distinctly higher dilutions of the sera obtained after immunisation than by the corresponding normal sera. Further, this table shows that in horse serum, as in that of all the other animals tested, the titre of normal agglutination is higher for U<sub>2</sub> than for X 19 as well as for the Kingsbury type of *B. proteus* X (XK) and for a strain of *B. typhosus* (No. 901) which is known for its particularly high sensitiveness to O agglutinins. Weltmann's globulin test was negative with the sera taken before the commencement of the immunisation and was strongly positive with the immune sera.

V. ANTIGENIC RELATIONSHIP BETWEEN *B. AGGLUTINABILIS* U<sub>2</sub>  
AND *B. COLI COMMUNIS*.

The high incidence of normal agglutinins for *B. agglutinabilis* U<sub>2</sub>, established with the serum of all the species tested, made it appear desirable to ascertain whether the O antigen of this particular strain was shared by other members of the *B. coli* group to an extent sufficient to account for the wide distribution of the corresponding antibody. Three high titre sera from rabbits immunised with *B. agglutinabilis* U<sub>2</sub> were tested against twenty-three strains of *B. coli* from the National Collection of Type Cultures. Twelve strains had to be



discarded owing to their "rough" condition; with the remaining strains the following results were obtained:

Four strains gave marked group agglutination with each of the three immune sera.

Two strains weakly reacted to two immune sera.

Five strains gave entirely negative reactions.

The six strains which did react to the immune sera originated from different localities and included Escherich's original strain of *B. coli communis*. This result seems to justify the conclusion to be drawn that the high incidence of normal agglutinins for  $U_2$  is due to community of antigen between this particular strain and numerous other members of the *B. coli* group.

## VI. DISCUSSION.

The observations recorded in this paper traverse those published by Wilson (1927). In my opinion the discordance is due to Wilson's failure to pay due attention to a sufficient number of adequately selected controls. The total number of non-typhus sera tested by Wilson against *B. agglutinabilis*  $U_2$  was forty-three and they were specified as follows:

Thirty-five sera were sent in for the Wassermann test.

Five sera were sent in for the Widal test.

Two sera from cases of scarlet fever.

One serum from a case of tuberculosis.

The habit of using as controls in agglutination tests sera which have been sent in for a Wassermann test, without knowing whether they give a negative or positive Wassermann reaction, is to be strongly deprecated because it is bound to lead to fallacious conclusions. Firstly, the majority of such sera generally give a negative Wassermann reaction and ought to be considered as "normal" and it has been emphasised in a previous section (p. 386) why normal sera cannot serve as adequate controls (Felix, 1918). Secondly, these sera are usually taken from the inactivated samples, left from the Wassermann test, and in doing so a very serious source of error is introduced since O agglutinins possess very low resistance to heat (Weil and Felix, 1917). Moreover, the globulin content of the serum markedly influences the heat destruction rate of agglutinins (Felix and Olitzki, 1929). Thus the usual procedure of heating the undiluted serum at 55° C. or 56° C. for  $\frac{1}{2}$  or 1 hour causes a considerable loss in the titre of O agglutinins and this loss is enhanced by the increase of globulins which generally coincides with a positive Wassermann reaction. It is obvious, therefore, that inactivated sera are useless in comparative agglutination tests.

Fletcher, Lesslar and Lewthwaite (1929) confirmed Wilson's results. They made control tests with *B. agglutinabilis*  $U_2$  and the blood of 478 persons who were not suffering from typhus. The description of the controls given by these workers reads as follows: "Most of these controls were hospital patients whose

blood had been sent to the laboratory for Wassermann or agglutination tests." Hence, their conclusion is invalidated for the same reason as that of Wilson.

It has already been stated by Wilson (1927) that typhus sera from Mexico failed to agglutinate  $U_2$  while reacting with X 19. From Table VIII it is seen that in my experience too the incidence of increased agglutinins for  $U_2$  was distinctly lower in cases of endemic typhus of the United States of America than in those of classical typhus from Poland. The viruses of Mexican Tabardillo and of endemic typhus of the United States share certain properties which distinguish them from European typhus virus (Mooser, 1928, 1929; Maxcy, 1929 *a, b, c*) and this is also reflected by differences in the reaction of patients' sera to various types of *B. proteus* X (Spencer and Maxcy, 1930; Felix and Rhodes, 1931). From an observation published by Havens (1927), who has had large experience with endemic typhus of the United States of America, a relevant inference can be drawn. Havens failed to confirm with American typhus sera that extreme heat-lability of X 19 agglutinins which numerous workers have established with patients' serum from European typhus (for references see Otto and Munter, 1930; Havens, 1927). In the light of our present knowledge the particular heat-lability of O agglutinins in typhus patients' serum, which markedly exceeds that of agglutinins produced by typhus virus in the rabbit, is most likely due to the globulin increase accompanying typhus infection in man (Felix and Olitzki, 1929). If, according to Havens, this heat-lability is not often met with in American typhus sera, absence of globulin changes is to be suspected as the most likely reason for this additional peculiarity of the American variety of typhus. This would account for Wilson's statement that Mexican typhus sera do not agglutinate  $U_2$ , an observation which accords with the results shown in Table VIII.

The same explanation suggests itself for the difference in reaction to *B. agglutinabilis*  $U_2$  established by Fletcher, Lesslar and Lewthwaite (1929) of the two serological varieties of tropical typhus occurring in the Federated Malay States. They found the agglutination reaction with  $U_2$  positive in the urban "W" form of tropical typhus, which corresponds to the X 19 type of *B. proteus* X, but negative with the rural "K" form of the disease, which is related to the Kingsbury type of *B. proteus* X. I have failed to confirm the conclusion arrived at by these workers, that the agglutination reaction with  $U_2$  affords an additional distinction between the two forms of tropical typhus. This reaction is neither uniformly positive with the "W" form (see Table VIII) nor is it invariably negative with the "K" form of typhus. However, the incidence of agglutinins for  $U_2$  is distinctly higher in the variety of tropical typhus which corresponds to type X 19 than in the "K" form of that disease or in tsutsugamushi, which two latter varieties of typhus react with the Kingsbury type of *B. proteus* X. It is suggested that the explanation for this difference is the same as in the case of American and European typhus.

From the analysis of the results recorded in Table VII it would appear that globulin increase is a necessary concomitant of increased heterologous

agglutination. However, the reverse of this relationship does not obtain. While the highest titres for  $U_2$  and Z 1 invariably coincide with a positive globulin test, the latter is not always accompanied by a high agglutination titre for these two polyagglutinable organisms. It is further seen from Table VII that none of the four highest titres for  $U_2$  coincides with any of the three highest titres for Z 1. Consequently the augmentation of the globulins cannot be the sole factor determining the increase in heterologous agglutination. It would appear that in order to produce that effect it is necessary for the increase of the globulins to occur in a serum endowed with a relatively large amount of normal agglutinins. It has been shown by Weil (1904) and is again demonstrated in Table X that summation effects can be obtained with a colloid and a specific immune agglutinin, when each is employed in concentrations incapable of producing *per se* a visible effect. The assumption appears to be justifiable that such a colloid will also enhance the action of preformed normal agglutinins.

It is not suggested that this is the sole mechanism involved in increased heterologous agglutination. However, in instances of polyagglutination like those of *B. agglutinabilis*  $U_2$  and *B. pyocyaneus* Z 1, the combined action of abundant normal agglutinins and augmented serum colloids may well account for the phenomenon.

An abundance of agglutinins is found in the normal serum of man and animals for organisms like diphtheroids, cocci, *B. coli*, *B. faecalis alcaligenes*, etc., which are living as saprophytes in the human and the animal body. Indeed, the polyagglutinable organisms described at various times are almost exclusively drawn from that category and *B. agglutinabilis*  $U_2$  is one of its typical representatives. In the case of *B. proteus* X 19, however, it cannot be shown that it is susceptible to the action of polyagglutinating sera. The fact that this organism reacts only with the serum of typhus patients cannot, therefore, be explained by the action of heterologous agglutinins.

#### SUMMARY.

1. In all species of animals tested—rabbit, guinea-pig, chicken and horse—normal agglutinins for *B. agglutinabilis*  $U_2$  are far more abundant than those for *B. proteus* X 19.

2. Human serum reacts in the same manner. Wassermann negative sera have been employed as normal sera. Their reaction in Weltmann's globulin test was also negative.

3. Wassermann positive sera from cases of syphilis and sera from lepers give distinctly higher agglutination reactions with  $U_2$  than normal human sera. On X 19, however, those sera do not exercise any increased agglutinating action.

4. Similar though somewhat stronger reactions with  $U_2$  are obtained with typhus sera. However, this reaction is far from being uniformly positive in typhus, while this is the case with the X 19 reaction.

5. The discordance between these results and those published by Wilson and by Fletcher, Lesslar and Lewthwaite is due to the failure by these workers to employ controls of suitable type.

6. In syphilis and leprous sera high titres of agglutination with  $U_2$  coincide with globulin-increase as indicated by the Weltmann reaction.

7. The summation effect of the non-specific agglutination by gelatine and the action of specific immune agglutinins, described by Weil, is again demonstrated.

8. The combined action of an abundance of preformed normal agglutinins and of augmented serum colloids is suggested as one of the factors involved in increased heterologous agglutination (polyagglutination).

9. *B. agglutinabilis*  $U_2$  is a typical representative of "polyagglutinable" organisms; *B. proteus* X 19, however, is not liable to the action of "poly-agglutinating" sera.

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## REFERENCES.

- ARKWRIGHT, J. A. (1921). *J. Path. and Bact.* **24**, 36.  
 BALLNER, F. and SAGASSER, R. (1904). *Arch. f. Hyg.* **51**, 245.  
 BAUER, K. (1921). *München. med. Wochenschr.* **68**, 1251.  
 BÜRGI, E. (1907). *Arch. f. Hyg.* **62**, 239.  
 CRUICKSHANK, R. (1927). *J. Hygiene*, **27**, 64.  
 DELTA, C. (1915). *Zentralbl. f. Bakt. Abt. I, Orig.* **76**, 50.  
 EPSTEIN, E. (1919). *Ibid.* **83**, 255.  
 EPSTEIN, E. and MORAWETZ, G. (1917). *Wien. klin. Wochenschr.* **30**, 399.  
 FELIX, A. (1917). *Zeitschr. f. Immunitätsf.* **26**, 602.  
 — (1918). *Wien. klin. Wochenschr.* **31**, 11.  
 — (1929). *J. Hygiene*, **28**, 418.  
 FELIX, A. and OLITZKI, L. (1929). *Brit. J. Exp. Path.* **10**, 25.  
 FELIX, A. and RHODES, M. (1931). *J. Hygiene*, **31**, 225.  
 FLETCHER, W., LESSLAR, J. E. and LEWTHWAITE, R. (1929). *Trans. Soc. Trop. Med. Hyg. London*, **23**, 57.  
 FRIEDBERGER, E., ZORN, W. and MEISSNER, G. (1922). *Zeitschr. f. Immunitätsf.* **34**, 259.  
 GIBSON, H. J. (1930). *J. Hygiene*, **30**, 337.  
 GOTSCHLICH, E., SCHÜRMAN and BLOCH (1915). *Med. Klin.* **11**, 1310.  
 HAVENS, L. C. (1927). *J. Infect. Dis.* **40**, 479.  
 KLAUSNER, E. (1908). *Wien. klin. Wochenschr.* **21**, 214.  
 KREUSCHER, A. (1918). *Berlin. klin. Wochenschr.* **55**, 374.  
 MAMLOK, L. (1909). *Arch. f. Hyg.* **68**, 95.  
 MARKL (1913). *Wien. klin. Wochenschr.* **26**, 1234.  
 MAXCY, K. F. (1929 a). *Publ. Health Rep. Washington*, **44**, 589.  
 — (1929 b). *Ibid.* **44**, 1735.  
 — (1929 c). *Ibid.* **44**, 1935.  
 MOOSER, H. (1928). *J. Infect. Dis.* **43**, 261.  
 — (1929). *Ibid.* **44**, 186.  
 NEGRE, L. and RAYNAUD, M. (1911). *Ann. Inst. Pasteur, Paris*, **25**, 619.

- NEUKIRCH, P. and KREUSCHER, A. (1919). *Beiträge z. Klinik d. Infektionskr.* **8**, 68.
- NICOLLE, C. and COMTE, C. (1910). *Bull. Soc. Path. exotique*, **3**, 214.
- OTTO, R. (1919). *Deutsche med. Wochenschr.* **45**, 817.
- OTTO, R. and MUNTER, H. (1930). In Kolle, Kraus u. Uhlenhut, *Handbuch d. pathog. Mikroorg.*, Jena, **8**, 1107.
- PALTAUF, R. (1904). *Ibid.* **4**, Part I, 668.
- (1913). *Ibid.* **2**, Part I, 483.
- PAPAMARKU (1915). *Zentralbl. f. Bakt. Abt. I, Orig.* **77**, 186.
- POSSELT and SAGASSER, R. (1903). *Wien. klin. Wochenschr.* No. 24.
- REICHENSTEIN, J. (1917). *Deutsche med. Wochenschr.* **43**, 554.
- RIZUTTI, G. and SCORDO, F. (1912). *Bull. Soc. Path. exotique*, **5**, 778.
- ROCHA-LIMA, H. DA (1919). *Erg. allg. Path. u. path. Anat.* **19**, 159.
- ROTKY, H. (1921). *Zentralbl. f. Bakt. Abt. I, Orig.* **87**, 16.
- SCHIFF, F. (1922). *Zeitschr. f. Immunitätsf.* **33**, 511.
- SPENCER, R. R. and MAXCY, K. F. (1930). *Publ. Health Rep. Washington*, **45**, 440.
- WEIGL, R. (1923). *Zeitschr. f. Hyg.* **99**, 302.
- (1924). *Klin. Wochenschr.* **3**, 1590, 1636.
- (1930). *Bull. Acad. Polonaise des Sciences et des Lettres, Classe de Médecine, Cracovie*, p. 25.
- WEIL, E. (1904). *Zentralbl. f. Bakt. Abt. I, Orig.* **37**, 98, 426.
- (1920). *Deutsche med. Wochenschr.* **46**, 343.
- WEIL, E. and FELIX, A. (1916). *Wien. klin. Wochenschr.* **29**, 974.
- — (1917). *Ibid.* **30**, 1509.
- — (1921). *Zeitschr. f. Immunitätsf.* **31**, 457.
- WELTMANN, O. (1916). *Wien. klin. Wochenschr.* **29**, 573.
- (1917). *Ibid.* **30**, 408.
- WILSON, W. J. (1909). *J. Hygiene*, **9**, 316.
- (1910). *Ibid.* **10**, 155.
- (1920). *Ibid.* **19**, 115.
- (1927). *Ibid.* **26**, 213.
- (1929). *System of Bacteriology*. Med. Res. Council, London, **4**, 318.
- WOLFF, G. (1922). *Ergeb. Hyg. Bakt.* **5**, 532.

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## THE MORTALITY OF A HERD OF MICE UNDER "NORMAL" CONDITIONS

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FOR the purpose of assessing the effects of exposure to a special environment on a herd of mice, one naturally desires a control series, viz. a herd not exposed to a specific risk but otherwise *in pari materia* with our colonies under experiment.

In 1928 one of us (Greenwood) published a study of two sets of mice not exposed to specific risk. These were sufficient to demonstrate a wide difference between herd mortality under favourable and unfavourable conditions, but the objection could be taken that in neither instance was the environment precisely the same as that of our herds at the London School of Hygiene and Tropical Medicine. We therefore decided to observe over a necessarily limited period a herd recruited, housed and treated in precisely the same way as our infected herds save that infected animals were at no time introduced.

This experiment was begun on 4. x. 1929 by the assembling of 20 normal mice; from 5. x. 1929 to 7. x. 1929 one mouse was added daily and from 8. x. 1929 to 17. i. 1930 three mice were added daily. The experiment was brought to an end on 27. v. 1930. In all 329 mice were under observation; of these 56 died. In 41 of these dead mice an autopsy was performed, and no

Table I.

Cage age in days	Expec- tation of life, unlimited	Expec- tation of life, limited to 60 days	Probability of dying in the next 5 days	Cage age in days	Expec- tation of life, unlimited	Expec- tation of life, limited to 60 days	Probability of dying in the next 5 days
0	205.08	56.79	0.0182	105	124.76	58.96	0.0035
5	203.83	57.37	0.0031	110	120.19	58.99	0.0070
10	199.45	57.12	0.0062	115	116.03	59.27	0.0
15	195.69	57.05	0.0031	120	111.03	59.15	0.0035
20	191.30	56.79	0.0063	125	106.41	59.23	0.0
25	187.49	56.72	0.0063	130	101.41	59.09	0.0037
30	183.67	56.69	0.0222	135	96.78	59.17	0.0038
35	182.81	57.65	0.0162	140	92.14	59.23	0.0
40	180.78	58.30	0.0066	145	87.14	59.04	0.0
45	176.96	58.42	0.0033	150	82.14	58.84	0.0045
50	172.55	58.37	0.0067	155	77.51	58.92	0.0050
55	168.71	58.52	0.0034	160	72.87	59.03	0.0053
60	164.27	58.47	0.0034	165	68.24	59.20	0.0
65	159.82	58.44	0.0068	170	63.24	59.04	0.0
70	155.89	58.62	0.0068	175	58.24	—	0.0
75	151.93	58.81	0.0034	180	53.24	—	0.0
80	147.45	58.83	0.0	185	48.24	—	0.0074
85	142.45	58.64	0.0	190	43.59	—	0.0088
90	137.45	58.45	0.0103	195	38.94	—	0.0
95	133.85	58.86	0.0069	200	33.94	—	0.0106
100	129.76	59.12	0.0				

*Mortality of a Herd of Mice*Table II. *Expectation of life, limited to 60 days.*

Cage age in days	Normal mice	Hill's mice (day 90 = day 0)	Murray's mice (day 0 = day 90)	Cage age in days	Normal mice	Hill's mice (day 90 = day 0)	Murray's mice (day 0 = day 90)
0	56-79	59-36	—	61	58-43	59-69	—
1	56-86	59-35	—	62	58-58	59-67	—
2	56-76	59-56	—	63	58-54	59-66	—
3	57-02	59-55	—	64	58-49	59-64	—
4	57-10	59-54	—	65	58-44	59-63	—
5	57-37	59-53	—	66	58-40	59-61	—
6	57-29	59-74	—	67	58-54	59-60	—
7	57-21	59-73	—	68	58-70	59-58	—
8	57-12	59-72	—	69	58-66	59-57	—
9	57-03	59-72	—	70	58-62	59-55	—
10	57-12	59-71	—	71	58-58	59-53	—
11	57-03	59-70	—	72	58-54	59-52	—
12	57-30	59-69	—	73	58-60	59-50	—
13	57-22	59-69	—	74	58-65	59-49	—
14	57-14	59-68	—	75	58-81	59-47	—
15	57-05	59-67	—	76	58-77	59-46	—
16	57-14	59-66	—	77	58-73	59-44	—
17	57-06	59-66	—	78	58-91	59-43	—
18	56-97	59-65	—	79	58-87	59-41	—
19	56-88	59-64	—	80	58-83	59-40	—
20	56-79	59-86	—	81	58-79	59-38	—
21	56-88	59-86	—	82	58-75	59-37	—
22	56-80	59-86	—	83	58-71	59-35	—
23	56-71	59-85	—	84	58-68	59-33	—
24	56-81	59-85	—	85	58-64	59-31	—
25	56-72	59-84	—	86	58-60	59-29	—
26	56-64	59-84	—	87	58-56	59-28	—
27	56-74	59-84	—	88	58-52	59-26	—
28	56-84	59-83	—	89	58-49	59-24	—
29	56-77	59-83	—	90	58-45	59-22	56-07
30	56-69	59-83	—	91	58-60	59-20	56-05
31	57-16	59-82	—	92	58-56	59-18	56-11
32	57-47	59-82	—	93	58-53	59-16	55-99
33	57-77	59-81	—	94	58-70	59-60	55-88
34	57-71	59-80	—	95	58-86	59-59	55-85
35	57-65	59-79	—	96	58-83	59-58	55-82
36	57-59	59-78	—	97	59-00	59-57	56-07
37	57-71	59-77	—	98	58-97	59-56	55-95
38	58-03	59-76	—	99	59-14	59-55	56-20
39	57-98	59-75	—	100	59-12	59-53	56-27
40	58-30	59-73	—	101	59-09	59-52	56-54
41	58-24	59-72	—	102	59-06	59-51	56-70
42	58-38	59-71	—	103	59-03	59-50	56-58
43	58-33	59-70	—	104	58-99	59-49	56-66
44	58-47	59-92	—	105	58-96	59-47	56-54
45	58-42	59-91	—	106	58-92	59-46	56-42
46	58-87	59-90	—	107	58-89	59-44	56-29
47	58-52	59-89	—	108	58-85	59-89	56-35
48	58-47	59-88	—	109	58-81	59-87	56-22
49	58-43	59-87	—	110	58-99	59-86	56-09
50	58-37	59-85	—	111	58-96	59-85	56-06
51	58-72	59-84	—	112	59-35	59-84	56-03
52	58-67	59-82	—	113	59-33	59-83	56-09
53	58-62	59-81	—	114	59-30	59-82	56-14
54	58-57	59-79	—	115	59-27	59-80	56-20
55	58-52	59-78	—	116	59-25	59-79	56-38
56	58-47	59-76	—	117	59-22	59-77	56-35
57	58-42	59-75	—	118	59-20	59-76	56-43
58	58-57	59-73	—	119	59-17	59-74	56-70
59	58-52	59-72	—	120	59-15	59-73	56-78
60	58-47	59-70	—				

disease was detected; 15 cadavera were not examined. The number of mouse days at risk was 53,906. Table I sets out: the probability of dying in the next 5 days, the complete expectation of life and the expectation limited to the next 60 days, at intervals of 5 days from day 0 to day 200 inclusive.

In Table II the expectations of life limited to the next 60 days of these mice and of the two series used by Greenwood are compared; the experience is limited to the first 120 days of observation. By day 200 the exposed to risk had dwindled to 96, and it seemed an unreasonable use of space to carry the study beyond the 120th day.

It has been assumed that day 90 of the Hill experience corresponds to day 0 of this experience, and that day 0 of the Murray experience corresponds to day 90 of this experience. The Hill mice were observed from birth, the Murray mice from the age of 6 months, while it is probable that our mice enter the herd at the age of about 3 months.

It will be seen that, although the experience of the Hill mice was somewhat more favourable than that of our control herd, the absolute difference is not very striking. We think, therefore, that this and the previous study provide a norm sufficiently exact for the purposes of our work which, for obvious reasons, must be mainly concerned with the first 50-100 days of herd life.

#### REFERENCE.

GREENWOOD, M. (1928). *J. Hyg.* **28**, 267-294.

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## A REVIEW OF THE CANCER STATISTICS IN ENGLAND AND WALES AND IN SCOTLAND BETWEEN 1891 AND 1927.

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(With a Diagram.)

THAT the number of deaths ascribed to cancer has steadily increased within recent years no one will deny, but as to the causes which have produced the increase there is not the same unanimity of opinion. Thirty years ago cancer did not rank very high in the list of fatal diseases. In 1899 the total number of deaths from cancer amongst persons in England and Wales was 26,325 as against 60,659 allocated to tubercular disease. Nowadays, "the old order changeth yielding place to new." According to the most recent statistics issued by the Registrar-General, in 1929, the number of deaths assigned to cancer was 56,896 and to all forms of tuberculosis 37,990. In view of this large increase in the number of deaths allocated to cancer it seemed of interest to review the cancer statistics of the last thirty years in this country and in Scotland. No investigation of this nature would be complete without first drawing attention to the very important work already done by Dr Stevenson in the *Annual Reports of the Registrar-General*, particularly the report for 1917 in which he examined the incidence of cancer in particular sites. The statistics of cancer in Scotland have not, until recently, received quite the same amount of attention as those of England. In a paper read to the Medical Association in Edinburgh and afterwards published in the *Journal* of that society, Dr Dunlop, the Registrar-General, gave a detailed account of the mortality, according to sites, between the years 1911 and 1928. He compared the actual numbers of deaths in 1920-2 and in 1928 with the numbers that might be expected to occur on the basis of the cancer mortality in age groups which prevailed in 1910-12. His method of analysis conforms partly to that of indirect standardisation. The conclusions as stated in the paper were:

I. There has, during recent years, been an increase in the cancer death-rate.

II. Of the increase the larger portion, approximately three-quarters, has been due to the ageing of the population leading to larger numbers living in those ages in which the cancer death-rate is high.

III. Subject to an exception in the case of mammary cancer the portion of the increase in the observed frequency unexplained by the ageing may

with reason be attributed to better recognition of cancer, and therefore does not indicate any true increase in the incidence of the disease.

The basis for the second deduction was afforded by the following figures taken from Dr Dunlop's paper:

Actual cancer deaths in 1911	...	...	...	= 5002
Actual cancer deaths in 1921	...	...	...	= 5953
Expected number of cancer deaths in 1921 on the basis of the death-rates at ages prevailing in 1911				= 5799
Therefore the expected difference expressed as a percentage of the actual difference	...	...		$\frac{797}{951} \times 100 = 83 \%$

The factor which is regarded as accounting for this 83 per cent. of the total increase in the number of cancer deaths does not measure a variation in the age constitution of the population only, its value depends upon:

- (1) the increase of the population between the two censuses;
- (2) the change in the age constitution of the 1921 census as compared with that of 1911.

If the object of an investigator is to measure the gross amount of cancer in a community, it is right to take into account all the deaths from cancer no matter at what age they occur. But if his purpose is to measure the rate of increase of cancer which is independent both of mere size of population and of age constitution, then it is essential that particular attention be paid to the ages at which the disease is prevalent. As 93 per cent. of the total cancer deaths occur after age 35 years it is practically irrelevant to any discussion of cancer mortality to introduce the population living before this period of life. A much more serious objection to the method is, however, the confusion of absolute increase and change of age constitution. Deductions drawn from the absolute numbers of deaths are misleading unless the population is stationary. The total population of Scotland in 1911 was 4,760,904 and in 1921 it amounted to 4,882,497 or increased approximately 3 per cent. If, however, we regard, and correctly so, the population at age 35 years and upwards as our "cancer population," we find that in 1911 there were 1,600,773 persons at this period of life and in 1921 1,821,626, an increase of 14 per cent. Hence it follows that if we are discussing the increase in the cancer mortality in Scotland as determined by age any conclusions drawn from an enumeration of the deaths alone are misleading. The deaths must be related to the population among which they occurred before any reliable deductions can be made. Taking the data in Dr Dunlop's paper and confining them to the ages 35 years and upwards, the results expressed in terms of the crude death-rates amongst persons are:

*Death-rate per 1000 persons aged 35 and over.*

	Actual	Expected
1911	3.008	—
1921	3.167	3.084

from which we observe that between 1911 and 1921 the mortality from cancer increased from 3.008 to 3.167 or 5.3 per cent. approximately. If there had been no change in the age constitution of the population the expected death-rate in 1921 would have been identical with the actual death-rate in 1911, but, in the present instance, there is a difference of 0.076 between them. In other words the age constitution of the 1921 population in comparison with that of 1911 had become unfavourable to a low cancer mortality. Expressing the increase 0.076 in terms of the total increase 0.159, that is the difference between the actual death-rates in 1921 and in 1911, we find that the change in age constitution of the 1921 population was responsible for 48 per cent. of the total increase in the cancer mortality and that other factors such as better diagnosis or a real increase in the disease accounted for 52 per cent. of the increased mortality and not 17 per cent. as suggested.

#### DATA.

For the purpose of the present enquiry the cancer mortality in triennial periods of which the central year is a census year has been calculated for the separate sexes beginning at 1890-92, *e.g.* the arithmetical average was taken of the number of deaths at specified ages occurring during the years 1890, 1891 and 1892 and afterwards related to the population enumerated at the 1891 census. The investigation ended with the year 1927, as the *Annual Report* for England and Wales, although since published, was not available when the analysis was made. In utilising triennial periods we avoid the errors which must inevitably arise when estimates of population are made for years removed from the census year. In the last period 1923-7, which was made a quinquennial one, there was no option but to use an estimated population, and the estimated population in 1925 was regarded as representing, on the average, the exposed to risk over this period. The mortality from cancer of particular sites classified into certain broad groups as accessible and inaccessible has been discussed and compared for the various triennia beginning at 1901. Proceeding on the lines previously stated the death-rates in this paper have been confined to the ages 35 years and upwards. Further, to eliminate any variation that may have occurred in age and sex constitution of the populations in the two countries, and to secure comparability of the total mortality at age 35 years and upwards, the death-rates have been standardised. The population utilised for this purpose was that enumerated in England and Wales at the census of 1901, this being the population frequently used as a standard for international comparisons of mortality.

In Table I the standardised death-rates amongst males and females are given for the two countries for the various triennia beginning at 1890-2 and also the successive death-rates expressed as a percentage of those obtaining in the first triennium. In England the mortality amongst males was 1.667 per 1000 in 1890-2, being much lower than that for females, 2.498, but there was a more rapid increase in male cancer, as, at the end of the period under

review, we find that male cancer was 83 per cent. in excess of that in 1890-2, whilst that for females increased only 20 per cent. In fact female cancer had been fairly stationary since 1900-2, when it was 13 per cent. higher than ten years earlier. During the two following triennia the excess remained fixed at 16 per cent. The rise in the cancer mortality in Scotland has been more pronounced than in England and Wales. In 1890-2 the mortality amongst males was 99 per cent. of that of English males, whilst that for females was only 88 per cent. During the quinquennium 1923-7 the position was reversed, as the Scottish death-rates amongst males and females were 8 per cent. and 13 per cent. respectively in excess of the corresponding values in England and were 98 per cent. and 55 per cent. higher than those which obtained thirty years earlier in Scotland.

In view of the fact that the mortality amongst males from cancer has almost doubled during the last thirty years and also that the increase amongst males is greater than that amongst females, it may well be asked whether the acceleration of the changes in cancer mortality has varied at particular intervals over this period. To afford the requisite information on this point the standardised death-rates at ages 35 years and upwards amongst males in England and Scotland were plotted for a series of triennia beginning at 1871 (Diagram I). The males were selected because their mortality has shown the greater increase. The distribution of the values suggests that the amount of increase has been on the whole practically constant for each period, in other words that the values are linearly distributed. The straight lines along which the death-rates are the most closely concentrated were obtained by the method of least squares. The expected values deduced from the resultant equations are given in conjunction with the actual values in Table II and also reproduced in the diagram. It will be observed that the agreement between fact and theory is close.

Although there has been a vast increase in the mortality ascribed to cancer, opinions differ as to whether it denotes a real increase in the disease itself or whether apart from the effect of the ageing of the population it is merely expressive of improved certification of the cause of death. As far back as 1893 this question of the increase of cancer was discussed by scientific investigators. In that year King and Newsholme in a paper on "The Alleged Increase of Cancer," the statistics for which were based on data for Frankfort-on-Main, showed that cancer of the accessible parts, likely to be more generally recognised, had not increased in the course of years, while inaccessible cancer had increased fairly considerably. These findings were later confirmed by Willcox who brought the original investigation in Frankfort up to a more recent date. Hoffman, on the other hand, has maintained in his work on *Mortality from Cancer throughout the World* that the increase was real. Since he utilised only crude death-rates, that is he made no allowance for any possible variability in the age constitution of the populations under consideration, his evidence cannot be accepted as wholly conclusive. In the Registrar-General's *Annual*

*Report* for 1926, Dr Stevenson draws further attention to this question of the increase of cancer as follows: "As classified in Table XLIV of the Review for 1926 practically half of the mortality of females in 1911-20 was from

Table I. *Showing the standardised death-rate per 1000 amongst males and females aged 35 years and upwards in England and Wales and in Scotland.*

Period	England and Wales				Scotland			
	Males		Females		Males		Females	
1890-2	1.667	100	2.498	100	1.657	100	2.188	100
1900-2	2.185	131	2.834	113	2.154	130	2.684	123
1910-2	2.606	156	2.890	116	2.649	160	3.159	144
1920-2	2.892	173	2.887	116	2.803	169	3.161	144
1923-7	3.052	183	2.989	120	3.277	198	3.388	155

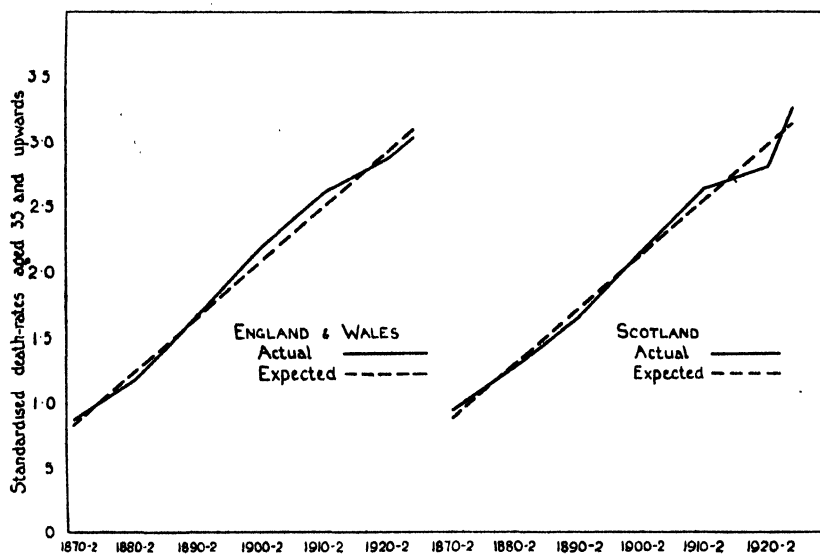


Diagram I.

Table II. *Showing the actual and expected death-rate from cancer amongst males aged 35 years and upwards in England and Wales and in Scotland.*

Period	England and Wales		Scotland	
	Actual	Expected	Actual	Expected
1870-2	0.861	0.836	0.941	0.888
1880-2	1.160	1.256	1.280	1.307
1890-2	1.667	1.676	1.657	1.726
1900-2	2.185	2.096	2.154	2.145
1910-2	2.606	2.517	2.649	2.563
1920-2	2.892	2.937	2.803	2.982
1923-7	3.052	3.105	3.277	3.150
	$y = 0.8358 + 0.4202 b$		$y = 0.8881 + 0.4188 b$	

cancer of accessible sites, but less than one-third that of males, and the increase since 1920 has been much greater in both sexes from growths of inaccessible than of accessible sites. If this change is to be explained as the result

of improvement in diagnosis it is evidently likely to apply chiefly to the sex providing the larger field for its application in the shape of inaccessible growths in which case the conversion of female into male excess of cancer mortality from 1924 onwards may well be apparent rather than real."

In view of this conflict of opinion it was deemed advisable to review the position as regards the mortality from cancer of particular sites over various periods. For this purpose the deaths in certain sites were classified into two main categories. It was thought better to treat the sites collectively rather than individually. By so doing we avoid any possible fluctuation in the mortality of particular sites due to a mere change of fashion in diagnosis such as the allocation of secondary manifestations to primary seats. The first group was composed of stomach, liver, oesophagus and intestines; the second comprised jaw, mouth, tongue, lip and rectum. Opinions may differ as to the inclusion of rectal cancer in the accessible group, but since Dr Stevenson assigns it to this group it was thought best to adhere to this classification. Cancer of the breast and uterus although assignable to the foregoing categories were treated separately. The initial period for which the statistics were analysed was taken as 1901-3 in England and 1902-3 in Scotland, as in the latter country no allocation of deaths to individual sites was made prior to 1902. The results are given in Table III.

Table III. *Showing the standardised death-rates per 1000 amongst males and females aged 35 years and upwards for certain sites classified as (1) inaccessible, (2) accessible, in England and Wales and in Scotland.*

		Inaccessible				Accessible				Uterus		Breast	
		Males		Females		Males		Females		Females		Females	
1901-3.	England and Wales	1.134	100	1.054	100	0.516	100	0.212	100	0.676	100	0.489	100
1902-3.	Scotland	1.168	100	1.141	100	0.410	100	0.169	100	0.467	100	0.388	100
1911-3.	England and Wales	1.379	122	1.193	113	0.635	123	0.224	106	0.604	89	0.532	109
	Scotland	1.550	133	1.477	129	0.497	121	0.203	120	0.533	114	0.475	123
1921-3.	England and Wales	1.428	126	1.143	108	0.653	127	0.229	108	0.533	79	0.560	115
	Scotland	1.622	139	1.513	133	0.551	134	0.176	104	0.492	105	0.510	132
1926-7.	England and Wales	1.510	133	1.186	113	0.652	126	0.225	106	0.506	75	0.579	118
	Scotland	1.850	158	1.594	140	0.573	140	0.178	106	0.523	112	0.553	142

*Inaccessible group.* We find that in England the most rapid increase in the mortality in the inaccessible sites occurred in the triennium 1911-13, as the mortality increased 22 per cent. amongst males and 13 per cent. amongst females. During the same interval in Scotland the rise has been more pronounced, and in the final period, 1926-7, the death-rate in this group exceeded that which existed twenty-five years earlier by 58 per cent. for males and 40 per cent. for females, the corresponding increments in England being 33 per cent. and 13 per cent. The contrast between the movements of the cancer mortality in this group amongst females in the two countries is worthy of notice—static in the one, steadily increasing in the other.

*Accessible group.* As regards the accessible group once again the most rapid movement occurred during 1911-13 when the mortality of males increased

23 per cent., but has remained at this level ever since; the increase amongst females was of smaller dimensions, *i.e.* 6 per cent. in the same period with little variability afterwards. In Scotland, on the other hand, the increase amongst males, though at first not quite so rapid as in England, continued, with the result that in 1926-7 accessible cancer was 40 per cent. greater than in the first period. The trend of the mortality amongst females was, apart from the high value during 1911-13, more or less identical with that in England.

*Uterine cancer.* As regards the seats of cancer which particularly affect women, breast and uterus, uterine cancer has steadily declined in England between 1901 and 1927 and, at the close of the period under review, the reduction amounted to 25 per cent. In Scotland the position has been quite different. Dr Dunlop has shown that in 1928 uterine cancer declined significantly as compared with 1911. It is interesting to note that this decline was not evident in Scotland up to 1927. During 1911-13 the mortality was 14 per cent. in excess of that in the previous period, between 1921-3 the death-rate declined, but still it was greater than that in 1902-3, and during the final period the excess was 12 per cent.

*Cancer of breast.* As regards cancer of the breast there has been a gradual increase in the two countries. In England the rate in 1926-7 was 18 per cent. higher than that prevailing twenty-five years earlier. In Scotland the increment has been more pronounced, as the mortality in our final period was no less than 42 per cent. above that which prevailed during 1902-3. To discover whether this decided increase, particularly in Scotland, was confined to any particular age period or was characteristic of all ages, the mortality in the individual age groups was examined and it was found that although the increase was fairly general it was more clearly defined at some ages. In the age group 35-45 the English mortality during 1926-7 was 14 per cent. greater than that in 1901-3, the corresponding excess in Scotland being 33 per cent. At ages 55-65 the relative position was much the same, whilst at ages above 65 the death-rate from cancer of the breast in England increased 23 per cent., the corresponding excess in Scotland being 50 per cent. It was at first thought that this increased mortality in Scotland might be attributable to the dilution of the female population owing to emigration from Ireland. If Irish women were prone to breast cancer this might offer a partial explanation. But it must be remembered that the Irish emigrant type invariably have large families, and as pointed out by Dr Stevenson in the *Annual Report* for 1913 breast cancer is usually more prevalent amongst the unmarried than the married women. To test the matter satisfactorily the mortality at ages from cancer of the breast amongst females in Ireland was calculated and it was found that the death-rates were lower than in either England or Scotland. Possibly hospitalisation of cases of breast cancer is carried out to a greater extent in England, and as surgical operation is a fairly successful line of defence in an illness of this description this procedure might afford a partial explanation of the divergency in the mortality between the two countries.

Reviewing the position as regards the increase of accessible and inaccessible cancer we find that in England there has been no appreciable difference between the amount of increase. In the 25 years under consideration the mortality amongst males in England from inaccessible sites increased 33 per cent. as against 26 per cent. for accessible parts. The corresponding rates for females were 13 per cent. and 6 per cent. respectively. In Scotland the increase amongst males for inaccessible cancer was 58 per cent. as against 40 per cent. for the accessible, but the increments for females were quite different as inaccessible cancer has increased by as much as 40 per cent., while the accessible has only advanced 6 per cent. Hence apart from the divergency for females in Scotland it is evident that the increase in the cancer death-rate is not easily attributable to a rising standard of diagnosis alone, but that some factor of actual increase in the disease, as revealed by mortality statistics between 1901 and 1927, has had its share.

## REFERENCES.

- DUNLOP, J. C. (1913). Increase in national cancer death-rate and its causation. *Edinburgh Med. J.* July.
- HOFFMAN, F. L. (1915). *The Mortality from Cancer throughout the World*. Newark, New Jersey: Prudential Press.
- KING, G. and NEWSHOLME, A. (1893). The alleged increase of cancer. *Proc. Roy. Soc.* **4**, 54. Registrar-General's *Annual Reports for the years 1913, 1917, 1926*.

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# APPEARANCE AND PERSISTENCE IN RABBITS' BLOOD OF RABICIDAL ANTIBODIES PRODUCED BY VARIOUS METHODS OF ANTI-RABIES IMMUNISATION

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(With a Chart.)

## INTRODUCTION.

AMONG the resolutions adopted by the Second and Third Commissions at the International Rabies Conference held in Paris during 1927 was one to the effect that comparative tests on a large scale should be carried out with anti-rabies vaccines killed by carbolic acid and by ether and that enquiries should be made into the rabicidal action of the serum of man and animals during and after immunisation. Such investigations were recommended by the conference, after due consideration of the results obtained by various recognised methods of treatment, in order that sufficient data might be made available for an accurate estimation of the comparative values of these methods. The present article deals primarily with a series of experiments performed to determine the relative content of rabicidal antibody possessed by the sera of several rabbit groups treated respectively with fresh-fixed virus, carbolised-fixed virus and etherised-fixed virus in equal quantities.

Before the main experiments could be undertaken it was obviously essential to carry out certain preliminary investigations and enquiries, the results of which have already been fully recorded by us elsewhere (1929); only brief reference, therefore, to the principal findings is necessary here.

### *(a) The nature of the antibody content of anti-rabies immune serum.*

It is generally agreed that specific changes in the blood serum characteristic of active immunisation follow treatment with anti-rabies vaccines and that those most likely to be evoked by stimulation of the animal organism with rabies virus itself are rabicidal, specific complement-binding and precipitating antibodies. Here, however, an inability to dissociate, for practical purposes, the antigen from its necessary vehicle of administration—nervous tissue—introduces a complication not normally encountered in immunisation problems. The antibody formation, under such circumstances, might well be both specific and non-specific: specific in response to the causal agent of rabies, non-specific in response to the nervous matter in the inocula—neurolytic and precipitating in nature. The conclusion reached by Schultz, Bullock and Brewer (1928)

that "no evidence exists for the presence of specific complement-fixing antibodies against the virus of rabies in the serum of rabbits immunised in various ways with fixed virus brains" has the support of the majority of workers including ourselves, who (1929) have shown that "degree and duration of anti-rabies immunity cannot be gauged by the Bordet-Gengou reaction." Again, Burmeister (1915) was unable to demonstrate the occurrence of specific precipitins in the sera of rabbits suffering from fixed virus rabies, and Lässer (1927) was equally unsuccessful in his efforts to adduce evidence of the formation of such precipitins in rabies-infected and in immunised animals. Broadly speaking, then, the position to-day is that, while it has not yet been finally decided whether the rabies virus *quâ* virus is wholly incapable of producing complement-fixing and precipitating antibodies in the immune organism, the occurrence of a rabicidal antibody is, from weight of experimental evidence, almost universally accepted as the sole serological effect of immunisation.

(b) *Rabicidal properties of immune serum.*

As considerable diversity of opinion exists in regard to the production of rabicidal antibody it became essential during our preliminary investigations to establish, if possible, proof of the existence and specificity of the antibody in immune serum, and of its absence in the sera of untreated animals and of animals treated with fresh normal brain. After considerable experimentation it was proved conclusively that rabicidal properties are non-existent in the sera of normal untreated animals and in the sera of animals treated with non-specific inocula such as normal nerve substance, whether homologous or heterologous; they are present, however, after specific stimulation with fixed virus emulsions. Proofs of existence and of specificity of rabicidal antibody having thus been advanced, there remained then to determine what relation the serum of an animal treated with fresh-fixed virus bears to that of an animal treated with carbolised virus and to that of one treated with etherised virus—all in equal amounts.

RECORD OF RABICIDAL PROPERTIES ACQUIRED AFTER IMMUNISATION.

Owing to the very large number of rabbits envisaged by such an experiment it was considered inadvisable to undertake the complete investigation at one time. The somewhat limited accommodation ordinarily available here for purely research laboratory animals together with the difficulty of obtaining a regular supply of rabbits compelled this decision. As a result the determination of rabicidal antibody consequent on treatment with fresh-fixed virus and with carbolised-fixed virus was made during 1929 (Experiment II, Part 1, Series *A*, *B* and *C*), that after treatment with etherised-fixed virus during 1930 (Experiment II, Part 2, Series *D* and *E*). This unavoidable division of the work necessitated the use of two control series of rabbits and unfortunately precluded, in the two parts of the investigation, the withdrawal of blood at

identical intervals after completion of the several forms of treatment employed. For reasons of brevity and clearness, however, the whole investigation is described as if it had been carried out during the same period of time.

### Experiment I.

*Proof that rabicidal antibody did not exist naturally in the serum of rabbits used in the main experiment.* Fifteen rabbits of 1400 gm. average weight were divided into three series *A*, *B* and *D*, each series including five animals. (Series *A* was later to be immunised with living fixed virus in N.S.S.<sup>1</sup>, series *B* with carbolised virus and series *D* with etherised virus.) All the animals were bled and their sera pooled for each series. 0.5 c.c. of serum *A*, 0.5 c.c. of serum *B* and 0.5 c.c. of serum *D* were then mixed respectively with quantities ranging from 0.125 to 0.5 c.c. of a 1:100 dilution of fixed virus and the serum-fixed virus mixtures exposed to a temperature of 37° C. for 2 hours. At the end of this time 0.2 c.c. of each mixture was inoculated subdurally into rabbits. A control series was also performed: 0.5 c.c. of a 1:100 fixed virus in N.S.S. was placed for 2 hours in the incubator at 37° C. and thereafter 0.2 c.c. was introduced subdurally into each of three rabbits. Table I shows the results obtained:

Table I.

Pooled sera of rabbit series	Test inoculum after 2 hours at 37° C. (0.2 c.c. subdurally)	Effects on rabbits of subdural inoculation	Rabicidal content of sera
<i>A</i>	0.5 c.c. serum + 0.125 c.c. of 1:100 F.V.	All died in 8 days	Nil
	0.5 " + 0.25 " "		
	0.5 " + 0.5 " "		
<i>B</i>	0.5 " + 0.125 " "	All died in 8 days	Nil
	0.5 " + 0.25 " "		
	0.5 " + 0.5 " "		
<i>D</i>	0.5 " + 0.125 " "	All died in 8 days	Nil
	0.5 " + 0.25 " "		
	0.5 " + 0.5 " "		
Control series	0.5 c.c. of 1:100 F.V.* in N.S.S.†	All died in 8 days	—
	0.5 c.c. of 1:100 F.V. in N.S.S.		
	0.5 c.c. of 1:100 F.V. in N.S.S.		

\* Fixed virus.

† Normal salt solution.

The death in 8 days of all rabbits inoculated with mixtures of normal serum + fixed virus in varying amounts proves, from the non-prolongation of the incubation period, the total absence of rabicidal antibodies in the sera of all three series of rabbits selected for later immunisation—first symptoms were invariably observed on the 5th day after subdural infection. Further, the experiment also shows from the control series that the fixed virus employed is not in any way affected by exposure to a temperature of 37° C. for 2 hours, 8 days being the normal killing time of the standard fixed virus used in all experiments here. (Our fixed virus in a 1:1000 dilution produces without exception symptoms of rabies on the 5th day with death on the 7th or 8th day.) The fixed virus emulsions referred to throughout this investigation were

<sup>1</sup> Normal salt solution.

in all cases prepared from the bulb of a freshly-extracted F.v. brain and the procedure of employing in all comparative tests the same anatomical part of a fresh brain is regarded by us as of first importance. The bulb is weighed and then thoroughly ground up in a sterile mortar; enough N.S.S. is gradually added to make an emulsion of 1:100 by weight. The emulsion is now filtered through four layers of gauze and the filtrate utilised to make up the various serum—F.v. mixtures used for rabbit inoculations. The mixtures are then incubated for 2 hours at 37° C. to permit of adequate union and 0.2 c.c. of each is inoculated subdurally into rabbits of 1400 grm. weight. This technique differs considerably from that advised by the International Rabies Conference of 1927, but appears to us equally satisfactory. A note describing the alternative procedure, however, is appended (see p. 422).

### *Experiment II.*

To guard against possible breakdown from fatalities among the animals undergoing immunisation, this, the main experiment, required the original employment of 15 rabbits, whose sera had been proved naturally free from rabicidal properties by the results detailed in Table I.

These rabbits were divided into three series *A*, *B* and *D*, each series consisting of five rabbits. Each rabbit in series *A* received on 14 consecutive days 5 c.c. daily of a 2 per cent. emulsion of fixed virus in N.S.S., each rabbit in series *B* received an identical dosage of killed carbolised virus and each rabbit in series *D* an identical dosage of killed etherised virus administered over the same period. In order that an accurate determination might be made of the true relative degree of rabicidal power possessed by the sera of rabbits immunised by the three methods, it was essential that one rabbit should be selected from each series and its blood taken at intervals after commencement and completion of treatment. The selection of the three representative rabbits entailed the laborious task of comparing the rabicidal power of the serum of each rabbit in each series during the earlier bleedings with that of the pooled serum of each rabbit group. The rabbit selected in each case was necessarily one whose serum showed identical rabicidal antibody content with the pooled serum of its own series. Fortunately all three rabbits ultimately chosen survived throughout the entire period of investigation.

The rabbit representing series *A* was bled 7, 14, 18, 22, 26, 34, 42, 67, 111, 129, 149 and 164 days after commencement of treatment, that representing series *B* 7, 14, 18, 22, 26, 34, 42, 67, 111, 129, 149, 164, 197, 218, 240 and 250 days after commencement, and that representing series *D* 7, 14, 18, 22, 46, 57, 70, 118, 144, 174, 204, 222, 240 and 256 days after commencement. One unit volume of undiluted serum was mixed with a varying number of unit volumes of a 1:100 suspension of fixed virus in N.S.S., and of these mixtures, after their exposure for 2 hours to a temperature of 37° C., 0.2 c.c. was introduced subdurally into rabbits. At the same time control series were

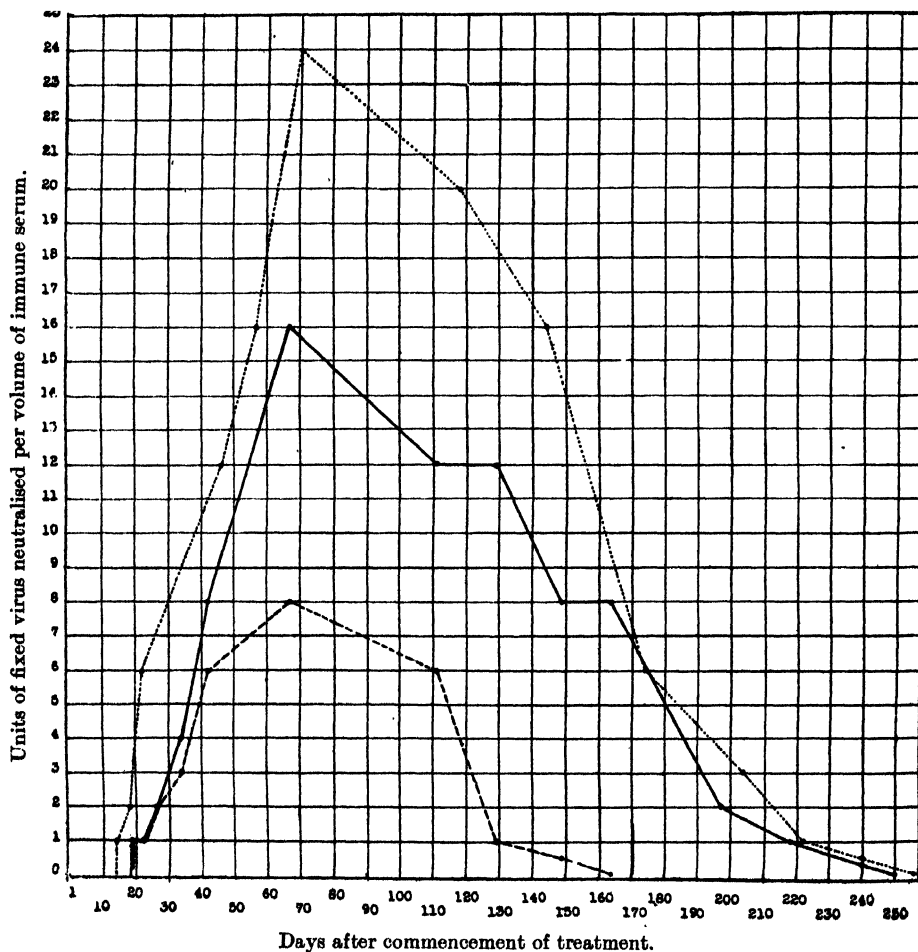
carried out with the serum of non-immunised rabbit groups, viz.: series *C* in respect of series *A* and *B*, series *E* in respect of series *D*.

Two rabbits were inoculated subdurally with each proportion of serum—F.V. mixture and in every case where the incubation period was unduly pro-

GRAPH COMPARING RABICIDAL PROPERTIES ACQUIRED BY RABBITS' SERUM  
AFTER IMMUNISATION BY VARIOUS METHODS

- (a) With fresh-fixed virus — continuous line.  
(b) With carbolised-fixed virus - - - - interrupted line.  
(c) With etherised-fixed virus ..... dotted line.

NOTE: One unit of fixed virus = 0.5 c.c. of a 1 % emulsion of F.V. in N.S.S.  
One volume of serum = 0.5 c.c. of undiluted immune serum.



longed or the symptoms in any way atypical, two rabbits were subpassaged with the brain in question to prove that death had been due to rabies.

As on the completion of the main experiment the findings proved somewhat unexpected, it was resolved to repeat on two further occasions the

**TABLE II.** *Experiments to show the relative content of rabicidal antibody possessed by the sera of various rabbit series treated with fresh-fixed virus, carbolised-fixed virus and etherised-fixed virus respectively.*

Rabbit series *A*: immunised with a 2 % emulsion of living fixed virus in n.s.s. in a dosage of 5 c.c. daily on 14 consecutive days.

Rabbit series *B*: immunised with a 2 % emulsion of killed carbolised virus in a dosage of 5 c.c. daily on 14 consecutive days.

Rabbit series *C*: not immunised and serving as controls for series *A* and *B*.

Rabbit series *D*: immunised with a 2 % emulsion of killed etherised virus in a dosage of 5 c.c. daily on 14 consecutive days.

Rabbit series *E*: not immunised and serving as controls for series *D*.

**PART 1. Series *A*, *B* and *C*.**

Serum and varying amounts of living fixed virus mixed and tested after 2 hours' incubation at 37° C.			Columns showing number of days after commencement of treatment when serum was tested, and results following subdural inoculation of rabbits with 0.2 c.c. of F.v.—serum mixtures prepared on these days. (— = rabbit lived; + = rabbit died)																
Serum tested	Amount of serum in c.c.	Amount of F.v. in c.c.	7	14	18	22	26	34	42	67	111	129	149	164	197	218	240	250	
Rabbit Series A	0.5	0.125	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	+	
	0.5	0.250	+	+	—	—	—	—	—	—	—	—	—	—	—	—	+	+	
	0.5	0.5	+	+	—	—	—	—	—	—	—	—	—	—	—	—	+	+	
	0.5	1.0	+	+	+	+	—	—	—	—	—	—	—	—	—	+	+	+	
	0.5	1.5	+	+	+	+	+	—	—	—	—	—	—	—	+	+	+	+	
	0.5	2.0	+	+	+	+	+	—	—	—	—	—	—	—	+	+	+	+	
	0.5	3.0	+	+	+	+	+	+	—	—	—	—	—	—	+	+	+	+	
	0.5	4.0	+	+	+	+	+	+	—	—	—	—	—	—	+	+	+	+	
	0.5	6.0	+	+	+	+	+	+	+	—	—	—	+	+	+	+	+	+	
	0.5	8.0	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	
Rabbit Series B	0.5	10.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	0.5	0.125	+	+	—	—	—	—	—	—	—	—	—	+	†	+	+	+	
	0.5	0.250	+	+	—	—	—	—	—	—	—	—	—	+	†	+	+	+	
	0.5	0.5	+	+	—	—	—	—	—	—	—	—	+	*	+	+	+	+	
	0.5	1.0	+	+	+	+	—	—	—	—	—	+	+	†	+	+	+	+	
	0.5	1.5	+	+	+	+	+	—	—	—	—	+	+	†	+	+	+	+	
	0.5	2.0	+	+	+	+	+	+	—	—	—	+	+	†	+	+	+	+	
	0.5	3.0	+	+	+	+	+	+	—	—	—	+	+	†	+	+	+	+	
	0.5	4.0	+	+	+	+	+	+	+	—	—	+	+	†	+	+	+	+	
	0.5	6.0	+	+	+	+	+	+	+	+	+	+	+	†	+	+	+	+	
Rabbit Series C	0.5	8.0	+	+	+	+	+	+	+	+	+	+	+	†	+	+	+	+	
	0.5	10.0	+	+	+	+	†	+	+	+	+	+	+	†	+	+	+	+	
	0.5	0.125	+	+	+	+	+	+	+	+	+	+	+	†	+	†	†	†	
	0.5	0.250	+	+	+	+	+	+	+	+	+	+	+	†	†	†	†	†	
	0.5	0.5	+	+	+	+	+	+	+	+	+	+	†	†	†	†	†	†	
* Incubation period 10 days.			† Incubation period normal.																

\* Incubation period 10 days.

† Incubation period normal.

**PART 2. Series *D* and *E*.**

Serum and varying amounts of living fixed virus mixed and tested after 2 hours' incubation at 37° C.			Columns showing number of days after commencement of treatment when serum was tested, and results following subdural inoculation of rabbits with 0.2 c.c. of F.V.—serum mixtures prepared on these days. (— = rabbit lived; + = rabbit died)													
Serum tested	Amount of serum in c.c.	Amount of 1:100 F.V. in c.c.	7	14	18	22	46	57	70	118	144	174	204	222	240	256
Rabbit Series D	0.5	0.125	+	—	—	—	—	—	—	—	—	—	—	—	—	+
	0.5	0.250	+	—	—	—	—	—	—	—	—	—	—	—	—	+
	0.5	0.5	+	—	—	—	—	—	—	—	—	—	—	—	+	+
	0.5	1.0	+	+	—*	—	—	—	—	—	—	—	—	+	+	+
	0.5	1.5	+	+	+	—	—	—	—	—	—	—	—	+	+	+
	0.5	2.0	+	+	+	—	—	—	—	—	—	—	+	+	+	+
	0.5	3.0	.	+	+	—	—	—	—	—	—	—	+	+	+	.
	0.5	4.0	.	.	+	+	—	—	—	—	—	+	+	.	+	.
	0.5	6.0	.	.	+	+	—	—	—	—	—	+	+	.	.	.
	0.5	8.0	.	.	.	+	+	—	—	—	—	+	+	.	.	.
Rabbit Series E	0.5	10.0	.	.	.	+	+	+	—	—	+	+	.	.	.	.
	0.5	12.0	.	.	.	.	+	+	—	—	+	+	.	.	.	.
	0.5	0.125	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.5	0.250	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.5	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+

\* One rabbit died after 18 days' incubation.

† Death occurred after 4 days' symptoms—subpassage proved rabies.

‡ Symptoms on 10th day: subpassage produced symptoms on 6th day and death on 10th.

process of immunisation with the three kinds of virus and of serum testing 7, 14, 18 and 22 days after commencement of treatment. The results in each case proved practically identical with those first obtained, and we feel justified, therefore, in our conclusions regarding the relative antigenic power of the viruses under test.

Preparation of the etherised virus was effected as follows: a fresh-fixed virus brain after having been weighed and cut into four pieces was placed in ether; after 90 hours' immersion it was removed and the ether allowed to evaporate under aseptic conditions; the nervous substance was then triturated in a sterile mortar, N.S.S. being gradually added till a 1:50 proportion was reached; finally the resultant emulsion was filtered through sterile gauze.

The results of our final investigations are shown in Table II, but the relative power of fresh-fixed virus, of carbolised-fixed virus and of etherised-fixed virus to evoke rabicidal antibody formation in the sera of immunised rabbits is more readily appreciated by reference to the Chart on p. 418.

#### SUMMARY.

A. Results following the subcutaneous inoculation of 1400 grm. rabbits with a 2 per cent. suspension of living fixed virus in a dosage of 5 c.c. daily on 14 consecutive days (1.4 grm.):

(1) Fresh living fixed virus introduced into rabbits subcutaneously produces in the sera of these animals so high a degree of rabicidal power that one unit volume of undiluted serum is capable of neutralising, when antibody formation is at a maximum, 16 unit volumes of a 1:100 suspension of fresh-fixed virus in N.S.S.

(2) Rabicidal properties in the serum are demonstrable 18 days after the commencement of treatment, one unit volume of serum then neutralising one volume of a 1:100 F.v. suspension in N.S.S.

(3) Rabicidal antibody content reaches a maximum between 50 and 60 days after completion of treatment, and thereafter diminishes somewhat gradually.

(4) Immune sera retain their rabicidal properties for a period extending over 222 days, viz. from the 4th to the 226th day after completion of treatment.

B. Results following the subcutaneous inoculation of 1400 grm. rabbits with a 2 per cent. suspension of killed carbolised-fixed virus in a dosage of 5 c.c. daily on 14 consecutive days (1.4 grm.):

(1) Killed carbolised virus administered to rabbits subcutaneously produces a very considerable degree of rabicidal power. The maximum reached, however, falls much short of that attained by immunisation with fresh-fixed virus and is only one-third of that following the use of etherised vaccine, one unit volume of serum neutralising 8 unit volumes of a 1:100 F.v. suspension in N.S.S.

(2) Rabicidal properties in the serum are demonstrable 18 days after the commencement of treatment, one unit volume of serum neutralising one unit volume of a 1:100 F.v. suspension in N.S.S.

(3) Rabicidal antibody content reaches a maximum some 50–60 days after completion of treatment, and thereafter subsides fairly rapidly.

(4) Sera of rabbits immunised with carbolised-fixed virus retain their rabicidal properties for a period of 131 days, viz. from the 4th to the 135th day after completion of treatment.

C. Results following the subcutaneous inoculation of 1400 grm. rabbits with a 2 per cent. suspension of killed etherised virus in a dosage of 5 c.c. daily on 14 consecutive days (1.4 grm.):

(1) Killed etherised virus introduced into rabbits subcutaneously produces in these animals' sera such an extraordinary degree of rabicidal power that, when antibody formation has reached a maximum, one unit volume of undiluted serum is capable of neutralising as many as 24 volumes of a 1:100 F.v. suspension in N.S.S.

(2) Rabicidal properties in the serum are demonstrable on the last day of treatment (14 days after commencement), one unit volume of serum neutralising one unit volume of a 1:100 F.v. suspension in N.S.S.

(3) Rabicidal antibody content reaches a maximum between 50 and 60 days after completion of treatment, and thereafter diminishes fairly gradually.

(4) Sera of rabbits immunised with etherised-fixed virus retain their rabicidal properties for a period of 226 days, viz. from the 14th to the 240th day after commencement of treatment.

#### CONCLUSION.

In the immune sera of rabbits treated with killed etherised-fixed virus, rabicidal antibodies make an earlier appearance, are present in greater degree and persist a longer time than in the immune sera of rabbits treated with equal quantities by weight of fresh-fixed virus or of killed carbolised virus.

#### REFERENCES.

- BURMEISTER, W. H. (1915). The absence of demonstrable specific antibodies in rabies caused by fixed virus. *J. Infect. Dis.* **17**, 423.
- LÄSSER, P. (1927). Zur Diagnose der Lyssa durch Präzipitation. *Zeitschr. f. Immunitätsforsch. u. exp. Therapie*, **53**, 1.
- SCHULTZ, E. W., BULLOCK, L. T. and BREWER, H. V. (1928). The antigenic properties of rabies virus. *J. of Immunology*, **15**, 265–281.
- STUART, G. and KRIKORIAN, K. S. (1929). Studies in Anti-Rabies Immunisation. *J. of Hygiene*, **29**, 1–34.



## NOTE (REFERRED TO ON P. 417).

Technique recommended by the International Rabies Conference, 1927. "Et pour permettre de mieux comparer les résultats obtenus par les différentes méthodes de traitement employées dans les Instituts antirabiques, elle recommande la technique suivante, non parce qu'elle soit la meilleure, mais parce qu'elle est la plus simple et en conséquence elle peut être mise en pratique par tous les Instituts:

A partir du 4ème et 5ème jour de traitement antirabique, tous les jours ou un jour sur deux ou trois, prélever par ponction dans une veine du pli du coude, 10 cent. cube de sang de façon à avoir 5 cent. cube de sérum. Faire d'autre part une émulsion à 1/100 de virus fixe, prélèvement au niveau du plancher du 4ème ventricule, passer celle-ci à travers un papier buvard et dans des flacons à hémolyse stérilisée, opérer des mélanges d'émulsion et de sérum en commençant par 5 parties de sérum pour 1 d'émulsion et en s'élevant jusqu'à 1 partie pour 2, 5, 10, 15, 20 parties d'émulsion, ces derniers taux (supposant un pouvoir antirabique déjà très élevé) n'ayant du reste besoin d'être atteints qu'à la fin du traitement ou quelque temps après lui.

Laisser en contact 24 heures à la glacière les mélanges de sérum et d'émulsion.

Après 24 heures, agiter, aspirer dans une seringue et inoculer 1/4 cent. cube du mélange sous la dure-mère du lapin ou du cobaye. Après avoir déterminé quand les substances rabicides apparaissent dans le sang au cours du traitement et quel taux elles atteignent, poursuivre ces recherches au moyen d'examen répétés les plus souvent possible pendant le mois qui suit le traitement. Essayer ensuite de déterminer quand elles disparaissent au moyen de prélèvements effectués chaque mois."

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## THE PHASES OF *HAEMOPHILUS PERTUSSIS*

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(With one Figure.)

### INTRODUCTION.

THE researches of Bordet and Sleswyck (1910) proved that agglutinating serums for the identification of freshly isolated strains of *Haemophilus pertussis* cannot be successfully made with old stock strains of the organism growing on agar, because of the serological change that occurs as the result of its adaptation to that medium. These workers considered the two serological states of the bacillus to be dependent on the medium of growth; state I being characteristic of cultures on fresh blood-medium and state II of growth on agar. The latter state was usually found to revert readily to the former when the culture was retransferred from agar to blood. In old stocks, however, a slow alteration to the second state tended to occur even on blood-medium, and toxicity to guinea-pigs was reduced or lost in the process (Bordet, 1913).

Krumwiede, Mishulow and Oldenbusch (1923) described two permanent serological varieties or types, A and B, of *H. pertussis* and were unable to confirm Bordet in his attribution of the two types to the nature of the culture medium. A close examination of their data, however, led us to doubt the validity of their conclusions. In Denmark, Kristensen (1922 and 1927) found the species to be serologically homogeneous, i.e. all cultures of his own isolation were of a single type. He admits the existence of Krumwiede's types but reserves his opinion as to their significance.

Finding it impossible to reconcile the conflicting views of these various workers, we embarked on a new investigation of the serological composition and variation of the species. Our analyses of thirty-two strains of *H. pertussis* by agglutination, absorption of agglutinins and tests of agglutinogenic properties have proved that they all fall into one or other of four well-marked agglutinative groups, which we call Phases I, II, III and IV.

Sixteen of the strains were freshly isolated by us, of which fifteen were in Phase I, and the sixteenth intermediate between Phases I and II. Four fresh Danish strains sent by Dr Kristensen were Phase I; one of them also showing some Phase II antigen.

All of our own strains that were tested, and all the Danish ones, were toxic to guinea-pigs.

Of seven strains from the Lister Institute, growing on egg-medium, three (nos. 760, 2471 and 2474) were in Phase III and four (nos. 366, 759, 364 and

761) in Phase IV. Six of these proved non-toxic; the seventh (no. 2474) not being tested.

Of five strains from the New York State Laboratories four were labelled Type B, and three of them proved to be Phase III and non-toxic. The fourth (no. 36), though agglutinating as a Phase III culture, had a strong toxic element which was identified by agglutination and absorption experiments with Phase II. The remaining strain, labelled Type A, was a non-toxic Phase IV. The classification of these American strains was confirmed by the testing of the American type-serums, of which "B" proved to correspond absolutely with our III and "A" with our IV.

#### TECHNIQUE.

(1) We have used the Danish modification of the original Bordet-Gengou medium, altered in a few respects. It is made as follows: potatoes are cleaned and cut into thin slices: 1 kg. of the slices with 2 litres of distilled water and 80 c.c. glycerine are boiled until the potatoes are soft. The water lost in the boiling is made up and the whole is filtered through linen. The juice is then adjusted to pH 7.0. One part of the neutralised juice and 3 parts of 0.6 per cent. NaCl are mixed and distributed in flasks; 200 c.c. in each. 4 per cent. agar is added and the flasks are left in the cold store overnight. The medium is then autoclaved, and to every 200 c.c. are added 4.5 c.c. of a sterile  $N/2$  solution of lactic acid (3.72 c.c. of ac. lact. of sp. gr. 1.21 to 100 c.c. of aq. dest.). When the flasks have been cooled to 45–50° C., 100 c.c. of fresh defibrinated horse's blood is added to each. The medium is then mixed well and poured into Petri dishes. It will keep good for a fortnight in the cold store.

(2) *Suspensions* were made in normal saline solution, to which 0.2 per cent. formalin had been added. In the case of cultures grown on Bordet-Gengou medium, the growth was scraped from the surface of the medium with a metal spatula and rubbed up in a small amount of normal saline solution in a mortar. On the whole the bacilli remained suspended extremely well, though it was sometimes necessary to shake thoroughly in order to obtain a good suspension. Salt sensitiveness was occasionally shown by cultures in Phase III when grown on Bordet's medium, but the phenomenon was very inconstant, for the next subculture on blood medium, or growth on egg, might be quite stable.

(3) Agglutinating serums were obtained by injecting formalised suspensions into the ear veins of rabbits. Two injections of 0.5 and 1.0 c.c. were given with a week's interval, and a blood-sample was taken on the fifth or sixth day after the last injection. If necessary, another injection was given, and the animal was bled after a further interval of 6 days. We have found that, generally speaking, two injections were sufficient to produce an agglutinating serum with a titre from 2000 to 5000.

(4) Agglutination tests were done with Dreyer's technique (Med. Res. Council 1920), and the tubes were incubated in the water-bath at 52–54° C.

Results could be read at  $4\frac{1}{2}$  hours, but a final reading was always taken after 18 to 24 hours. The last definite trace read with a hand-lens after incubation overnight was taken as the end-point of the reaction.

All suspensions were tested as a routine with antiserums for Phases I, III and IV, and if necessary other serums, *e.g.* Phase II, were included. The sensitivity of various suspensions varies very little, except for the occasional reduced sensitivity of Phase IV when grown on Bordet's medium. In the case of anomalous results, *e.g.* a supposed Phase IV suspension agglutinating to full titre with a Phase III serum, subsequent examination of the culture by plating and colony pickings has shown both phases to be present.

(5) Absorption tests were carried out in the usual manner. The quantities of bacilli used were in the region of 8.0 mgm. of moist culture to 1.0 c.c. of serum, diluted either 1:20 or 1:40 according to its titre. Thus, three well-grown Petri dishes of Bordet's medium emulsified in 10–12 c.c. of saline made a suitable suspension, to which the serum was added in the requisite amount. The absorption and control mixtures were incubated in corked tubes at  $37^{\circ}\text{C}$ . for  $2\frac{1}{2}$ –3 hours, left at room temperature overnight and then centrifuged.

#### THE RELATIONSHIPS OF THE FOUR PHASES.

In Fig. 1 are given the averages of a large number of experiments in which the constancy of the results was remarkable; the slight variations of titre that were observed being probably due to differences in the sensitivity of the suspensions. The Phase I serum (no. 1) was made from a suspension of the colonies on a cough-plate which showed almost a pure growth of *H. pertussis*. It agglutinates Phase III very little compared with Phase I. It must, however, be mentioned that another serum (no. 2), made with the same strain after cultivation on Bordet's medium for some months, had as high a titre for Phase III as for Phase I. Another serum made a little later had a similar character. We have used Phase I, no. 1 serum throughout the whole of this work, since it distinguished the phases so sharply. The characters of the numerous other Phases II, III and IV serums, made for various purposes, agreed in all essential points with those shown in the figure.

Absorption tests were done from time to time to confirm the agglutination results. In Table I are given the results of some experiments to determine the cross-absorptive powers of Phases I, II, III and IV suspensions. For the sake of brevity only one representative set of experiments is given, though the results have been confirmed on other occasions with different strains.

The chief points demonstrated are: that Phase I agglutinins are untouched by absorption with Phases II, III and IV. Phase I reduces the homologous titre of Phase II serum and removes Phase III agglutinins from all the phase serums. It is likely that freshly isolated Phase I bacilli would have less, if any, affinity for the Phase III antibody. Phases I and III have a definite though partial absorptive action on Phase IV serum, and finally Phase IV has little or no action on heterologous serums.

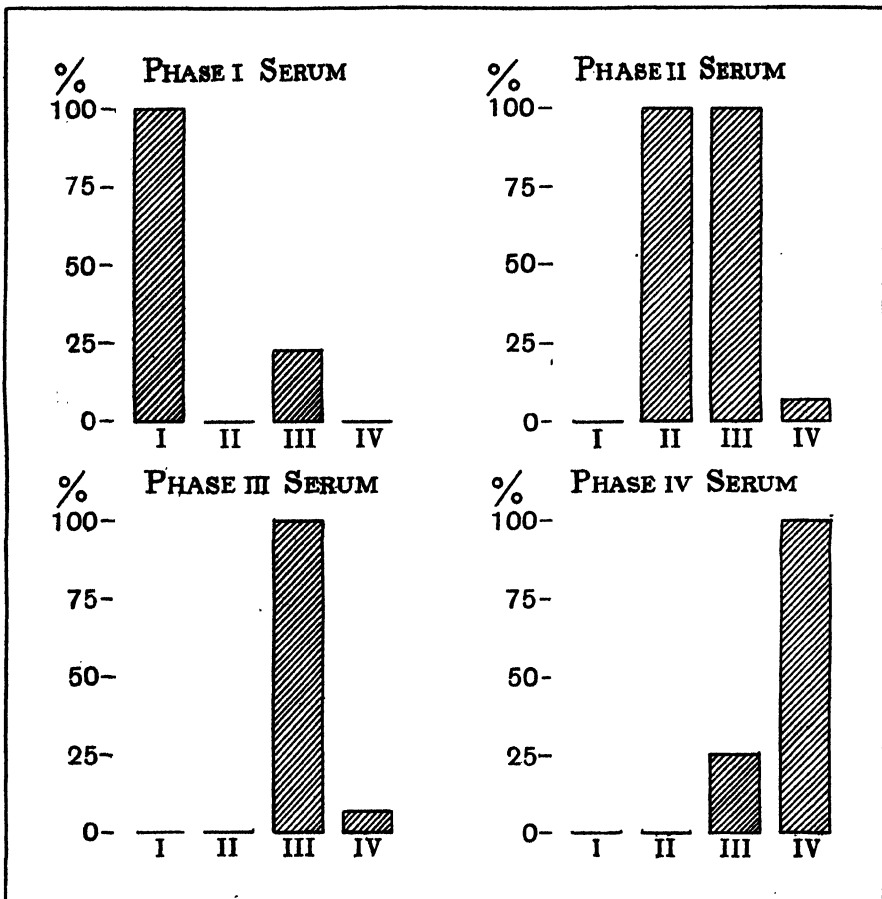


Fig. 1. *Cross-agglutination Tests with Phase Serums.* Minor agglutinins expressed as percentages of the titre to the homologous serum-strain.

Table I. *Cross-absorption tests with Phases I, II, III and IV.*

Serum	Organism agglutinated Phase and Strain	Titre before absorption	Titre after absorption with			
			Phase I (H.W.)	Phase II (Am. 36)	Phase III (2471)	Phase IV (364)
Phase I, No. 3	I (H.W.)	4000	100	—	4000	4000
	I (Ruth)	2000	—	1000	—	—
	III (2471)	2000	<100	—	<100	1000
Phase II	II (H.W.)	2-4000	500	50	4000	—
	II (Am. 36)	5000	—	<50	—	—
	III (2471)	4000	<100	—	<100	—
Phase III	III (2471)	1000	50	—	<50	1000
Phase IV	III (2471)	500	<100	—	<100	<100
	IV (364)	1000	400	—	300	<100

All cultures were grown on Bordet's medium and the absorption doses were in the region of 7-8 mgm. of moist culture per c.c. of diluted serum.

Comparing these results with Bordet's and Krumwiede's records, we find that they are all in accord if Bordet's first serological state corresponds with our Phase I, and if his second state, together with Krumwiede's type B, is our Phase III. Krumwiede's type A corresponds completely with our Phase IV.

#### TRANSFORMATION OF PHASES.

By transplantation on to appropriate media, addition of immune serum to a fluid substrate, selection of colonies and other simple procedures, we have shown that a culture derived from a single colony can produce in turn all the antigens characteristic of the four phases. Moreover, the change is to a considerable degree reversible, though there is evidence that Phase IV may become firmly and irrevocably established in some cultures. The details of the experiments are sufficiently shown in Table II.

It was in the course of these experiments that we first recognised the antigen characteristic of Phase II, and therefore an agglutinating serum for it was available only in the later stages. This phase does not seem to occur regularly as an intermediate stage between I and III, and many points about it remain obscure; but we have had no difficulty in proving its reality by the usual cross-agglutination and absorption tests (Table I and Fig. 1). It is toxic and tends to undergo transformation to Phase III.

#### THE SIGNIFICANCE OF THE TRANSFORMATION OF PHASES.

We believe that Phases I and II correspond to the smooth or pathogenic form of other bacteria, Phases III and IV to the rough, relatively harmless, saprophytic form. Under cultivation there is a general tendency to pass from the smooth to the rough state, but the addition of sufficient fresh blood to the medium makes it possible for the parasitic phase to persist unaltered for long periods. This accounts for Kristensen's belief in the serological uniformity of the species, for his strains were grown and stored exclusively on Bordet's medium. It appears from the work of Debré, Marie and Pretet (1928) that small quantities of blood in the medium are not so efficacious, for their cultures isolated a year previously were agglutinated by the American type B serum, *i.e.* had changed to Phase III.

Of our "rough" phases, no. IV is the more complete, being apparently irreversible in some cultures. Phase III may be considered as a transitional or partial rough, with the power either to revert to I or pass on to IV.

The direct, visual qualities of roughness and smoothness are not very obvious in *H. pertussis*, nor is there any constant and measurable difference in salt-sensitiveness. But there is no doubt that Phases III and IV are on the average rougher and have a greater tendency to instability in NaCl solutions than Phase I.

Exp.	Strain	Course of cultivation on media named	phase serums				Remarks
			I	II	III	IV	
1	H.W.	Bordet (single colony)	100	0	0	0	Isolated by us 1929. Toxic to guinea-pigs (Table III)
		subcultured Bordet 18 months	100	0	0	0	
	s.B. (3 months at R.T.)						
		s.B. (4 months at R.T.)					
		Bordet (3 colonies agglutinated)	100	—	0	0	
	s.B. (1 generation)						
	s.A.		25-50	—	0	0	No Phase II serum available
		s.A. several generations	50-100	—	0	0	
		Egg (3rd subculture)	15-20	—	100	20	Agglutinogenically Phase III and non-toxic to guinea-pigs (Table IV)
		Egg (1 colony on old slope at R.T.)	0	0	0	100	
		2nd subculture agglutinated					Agglutinogenically Phase IV and non-toxic to guinea-pigs (Table V)
2	H.W.	Bordet (single colony)	100	0	0	0	Toxic to guinea-pigs (Table III)
		5 % Phase I immune s.B. (2 subcultures)					Agglutinogenically Phase III. Very early experi- ment, when no Phase III serum was available
		s.B. (6 days' growth)	20	—	—	25	
		5 % Phase I immune s.B. (3 subcultures)	0	50	0	0	Agglutinogenically Phase II
		1 colony on s.A.					
		Egg (8 subcultures)	20	—	100	25	
3	Holland	Broth and agar (several single colonies agglutinated)	20	—	100	25	
		Bordet (single colony)	100	0	0	0	Isolated by us 1929. Toxic to guinea-pigs (Table III)
		5 % Phase I immune s.B. (3 subcultures)					
		s.A. (single colony)	0	100	0	0	
		1 colony					
		Egg (2nd subculture)	20	—	100	25	
4	Lister 761	Bordet (single colony)	0	—	0	100	Received on egg from Lister Institute; ob- tained from America 1920
		Bordet (10 daily subcultures)	0		0	100	
		s.B. (24 hours)					Confirmed by absorption test
		s.A. (2 generations)	20	—	100	50	Confirmed by absorption test
5	Lister 761	Bordet (single colony)	20	—	100	25	
		s.B. (24 hours)	0	—	0	100	
		Bordet (plating)					
		3 colonies agglutinated	0	—	0	100	Very salt-agglutinable suspension-readings only approximate
		1 colony	? 25	—	? 100	? 25	
		Egg	50	—	100	25	Non-toxic to guinea-pigs (Table IV)
6	Lister 2471	Egg	25	—	100	25	Received on egg from Lister Institute, isolated 1927
		Bordet (plating)					
		majority of colonies	25	—	100	25	Non-toxic to guinea-pigs (Table IV)
		1 colony	100	—	0	0	Toxic to guinea-pigs (Table III)
			20	—	100	25	Second generation on egg proved non-toxic to

A NOTE ON THE RELATIONSHIP BETWEEN *H. PERTUSSIS* AND  
*B. BRONCHISEPTICUS*.

Ferry and Noble (1918) have described a one-sided serological relationship between *B. bronchisepticus* and *H. pertussis*, in that an antiserum of the former agglutinated suspensions of the latter organism to a considerable extent, whereas their *pertussis* antiserum had no effect upon suspensions of *bronchisepticus*. Further, normal serums of a rabbit stock which had an epidemic due to the latter organism agglutinated *pertussis* sometimes up to a titre of 1:800.

We have found that an agglutinating serum made from a strain of *bronchisepticus* (N.T.C. Man) reacted on our *pertussis* Phase IV to 20–100 per cent. of the homologous titre, and on Phase III to 5–25 per cent.; the suspensions varying greatly in their agglutinability by this serum. *Bronchisepticus* suspensions were agglutinated partially by Phase IV serums and were completely unaffected by all our other phase-serums. Evidently there is a close serological relationship between this strain of *bronchisepticus* and our *pertussis* Phase IV. Since *bronchisepticus* infection is unknown in our animal houses we are satisfied that no error due to this infection has affected our serological results.

TOXICITY EXPERIMENTS.

With the two exceptions mentioned in the tables all strains tested for toxicity were grown for 48 hours on Bordet's medium in Petri dishes after sufficient subcultures had been made to induce free growth. The bacilli were removed from the surface of the medium, weighed wet, suspended in either saline or Ringer's solution, and injected intraperitoneally into guinea-pigs as soon as possible.

The doses were given in milligrams irrespective of the weight of the animal, and were subsequently calculated in relation to the blood volume of each animal, according to Dreyer's (1911) formula, thus:

$$\frac{\text{Dose in mgms.} \times 1000}{(\text{Weight in grams})^{0.72}}.$$

The results are expressed as whole numbers.

In Tables III and IV the results of injection of Phases I, III and IV are given. In the case of the type strain (H.W.) in Phase I it will be seen that, when the doses are arranged in decreasing amounts, a point is reached where some of the animals live and others die. The same effect is shown by our own recently isolated strains. This dose, which may be termed the approximate M.L.D., lies in the region of 25–30. Bordet's view that the death is purely toxic and that there is no multiplication of the culture within the guinea-pig is fully corroborated by our observations. The percentage loss of weight, calculated for all animals that lived for four days or upwards, is roughly propor-



tional to the size of the dose. The post-mortem changes in the animals that died from toxæmia varied greatly according to whether the death was acute or delayed. In those dying within three days the changes were similar to those described by Bordet, viz. blood-stained peritoneal effusion with petechial hæmorrhages in the peritoneal wall, commencing necrosis in the liver and intense injection of the testicles and sometimes of other organs, *e.g.* the spleen. In our experience the pleural effusion described by Bordet has not occurred frequently. When death is delayed for 30 days these acute reactions have

Table III. *Toxicity experiments with Phase I cultures.*

Type strain H.W.					Other Phase I strains					
	Guinea-pig weight	Dose	Death in days	% loss in weight	Sex	Strain	Guinea-pig weight	Dose	Death in days	% loss in weight
♂	206	87	2	—	Own recently isolated strains:					
♂	312	64	Lived	0*						
♂	320	63	2	—	♂	Hart	245	76	1	—
♂	336	61	5	35	♂	Holland	245	76	2	—
♂	350	59	6	31	♂	McMahon	247	76	2	—
♂	353	59	2	—	♂	McMahon	229	60	1	—
♂	415	58	2	—	♂	Hart	271	36	9	26
♂?	360	58	6	27	♂	Holland	280	35	7	28
♂	361	58	6	40	♂	Mace	325	31	8	33
♂	363	58	2	—	♂	Christine	445	25	7	38
♂	368	58	2	—	♂	Low	561	21	Lived	37
♂	372	56	2	—	♂	Low	576	21	Lived	20
♂	375	56	2	—	♂	Low	584	10	Lived	9
♂	376	56	2	—	Danish strains:					
♂	380	55	5	26						
♂	382	55	2	—	♂	E. Chr.	229	80	2	—
♂	400	54	3	—	♂	Else†	230	80	4	—
♂	443	50	6	33	♂	Ruth	252	75	1	—
♂	464	48	13	41	♂	1778	256	74	2	—
♂	473	47	2	—	♂	E. Chr.	222	61	Lived	0
♂	401	27	Lived	29	♂	Else	226	61	7	27
♂	412	26	26	38	♂	Ruth	228	60	2	—
♂	432	25	10	37	♂	1778	243	57	Lived	38
♂	264	18	Lived	22	Reverted strains:					
♂	403	13	Lived	18						
♂	238	5	Lived	0	♀	Lister 2471	263	72	3	—
♂	267	1	Lived	0	♂?	Lister 2471	326	62	11	27
					♂	Lister 2471	260	37	Lived	23

Dose =  $\frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0.75}}$  expressed as nearest whole number.

\* Subsequently injected with a dose of 59—death in 3 days.

† "Else" was a mixture of Phase I and a small amount of Phase II.

cleared up, but there is evidence of extensive damage to the liver in the shape of numerous focal necroses in all stages of degeneration and repair.

Table IV shows that guinea-pigs tolerate a twenty or thirty times larger dose of Phases III and IV than of Phase I. We have therefore felt justified in using the term toxic for the latter, and non-toxic for the former, although it is not impossible that Phase III may possess a residue of toxicity, demonstrable perhaps in other animals or by some more delicate method.

Table IV. *Toxicity experiments with Phase III and IV cultures.*

	Guinea- pig sex	Strain	Guinea- pig weight	Dose	Result	% loss in weight
<b>PHASE III.</b>						
Lister strains:	♂	2471	243	610	Lived	11
	♂	760	240	290	Lived	10
American Type B strains:	♂	2471	272	140	Lived	0
	♂	35	199	440	Lived	9
	♂	34	317	320	Lived	10
	♂	33	344	300	Lived	7
	♂	35	193	230	Lived	0
	♂	34	315	160	Lived	0
	♂	33	353	150	Lived	5
Transformed strains:	♂	Lister 761	190	460	Lived	2
	♂	H.W.	340	300	Lived	19
	♂	Lister 761	214	210	Lived	0
	♂	H.W.	362	140	Lived	3
	♂	Lister 2471	274	70	Lived	7
	♂	Ph. 1-111 (Egg)	332	31	Lived	2
<b>PHASE IV.</b>						
Lister strains:	♂	364	242	770	Lived	0
	♂	366	259	370	Lived	0
	♂	759	259	370	Lived	12
	♂	364	300	260	Lived	0
Transformed strains:	♂	H.W.	320	310	Lived	10
	♂	H.W.	338	150	Lived	3

$$\text{Dose} = \frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0.72}}$$

## IMMUNITY EXPERIMENTS.

The following experiments were performed in order to discover whether it is possible to protect guinea-pigs by means of vaccines against a lethal dose of living Phase I organisms, and also whether there is any difference in the immunising powers of the various phases. All vaccines and test cultures were grown on Bordet's medium and their serological state was determined by agglutination. The normal animals used as controls are all included in Table III (strain H.W.).

*Exp. no. 1* was the comparison between killed Phase I and living Phase III vaccines. A Phase I vaccine (strain H.W. 4.0 mgm. per c.c.) was divided into two portions; one killed by heating to 55° C. for ½ hour, the other killed in the cold with 0.4 per cent. formalin. Both were tested for sterility before use. Phase III vaccine consisted of a living suspension of strain 2471, 4.0 mgm. per c.c. Animals received subcutaneously two injections of the same dose, with a 7 days' interval; and 14 days after the second a test dose of living Phase I organisms (strain H.W.) was given intraperitoneally. The results are shown in Table V. The twelve guinea-pigs that had received Phase I vaccine survived, while those injected with Phase III living vaccine showed no immunity at all. None of the controls survived.

In order to compare our phase vaccines with those on sale for human use, some prophylactic *B. pertussis* vaccine was obtained from Parke Davis and Co. It was found to be serologically in Phase III.

*Exp. no. 2.* Vaccines of Phases I, III and IV were made by suspending

the bacilli in physiological saline to which 0.2 per cent. of formalin had been added. They were then diluted to the same opacity as the Parke Davis vaccine and were left in the cold store, being shaken daily until sterile.

Table V. *Immunity experiment no. 1.*

Phase I vaccine:					Phase III vaccine (living):				
Nature	Two doses of mgm.	Guinea-pig weight	Test dose	% loss in weight	Result	Two doses of mgm.	Guinea-pig weight	Test dose	Death in days
Heated 55° C.	2.0	320	63	Lived	31	1.0	292	67	4
"	1.0	359	58	"	33	2.0	311	64	2
"	4.0	369	57	"	29	4.0	354	59	5
0.4 % formalin	4.0	366	57	Lived	38				
"	1.0	377	56	"	19				
"	2.0	397	54	"	26				
"	4.0	309	32	"	8	4.0	304	33	14
"	1.0	328	31	"	22	2.0	352	29	14
"	2.0	362	29	"	17	1.0	385	28	46
Heated 55° C.	4.0	357	29	Lived	13				
"	1.0	378	28	"	11				
"	2.0	403	27	"	0				

All the guinea-pigs were females.

Dose =  $\frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0.75}}$

expressed as nearest whole number.

\* Killed in a moribund condition.

The animals received an initial dose of 0.25 c.c. subcutaneously followed at intervals of 6 and 5 days by doses of 0.5 and 1.0 c.c. respectively. Twenty-one days after the last injection they received a test dose of a living Phase I (strain H.W.) suspension intraperitoneally.

The results are given in Table VI. None of the animals vaccinated with Phases III and IV or with the Parke Davis vaccine survived, but of the four animals which received Phase I two lived. The remaining two and all the controls died.

*Exp. no. 3* was similar, except that other strains were used for making the vaccines, which, as before, were made up in physiological saline solution containing 0.2 per cent. of formalin to the same density as the Parke Davis vaccine. The animals received an initial dose of 0.25 c.c. followed at intervals of 5 days by 0.5 and 1.0 c.c. Twenty-two days after the last injection they received a test dose of a living Phase I (strain H.W.) suspension intraperitoneally.

The results are given in Table VI. Out of eight animals vaccinated with the Phase I suspensions two survived. All those receiving the Parke Davis and the Phase III vaccine, as well as the controls, died.

Summarising the last two experiments: out of twelve guinea-pigs vaccinated with Phase I four survived, and of nineteen animals treated either with Parke Davis vaccine or with Phases III and IV none survived. Eight control animals also died.

A further experiment was done by immunising ten animals with a vaccine made with a recently isolated Phase I strain. These, with an equal number

of controls, were then injected intraperitoneally with a dose of 55 of the same toxic strain (H.W.) used in the previous experiments. The experiment was a failure, since out of the ten controls, four survived with no loss of weight. This was completely opposed to our previous experience in which out of a total of twenty-seven animals injected with our own toxic strains in doses greater than 30, only one had survived. Whether this increase in percentage of animals unaffected by a toxic dose of this strain is an indication of a commencing loss of Phase I antigen, we are unable to say. Although the strain had been under cultivation for some 18 months, it was still serologically in Phase I and we had no reason to expect a falling off in toxicity. Of the immunised group of animals just as many died as of the controls, so that no evidence of immunity was obtained.

Table VI.

*Immunity experiment no. 2.*

Vaccine. Doses: 0.25, 0.5, 1.0 c.c.

Guinea-pig sex	Strain	Phase	Guinea-pig weight	Test dose	Death in days	Loss in weight
♂	Low	I	314	64	Lived	0*
♂	"	"	362	58	6	30
♀	"	"	369	57	2	—
♀	"	"	381	56	Lived	0†
♂	2471	III	395	54	2	—
♂	"	"	400	54	3	—
♂	"	"	447	49	3	—
♂	"	"	452	49	3	—
♂	Parke Davis	III	420	52	3	—
♀	"	"	426	51	31	53
♀	"	"	449	49	23	48
♀	"	"	↓	—	—	—
♂	364	IV	380	56	2	—
♂	"	"	400	54	2	—
♂	"	"	411	52	2	—
♀	"	"	431	51	2	—

*Immunity experiment no. 3.*

Vaccine. Doses: 0.25, 0.5, 1.0 c.c.

Guinea-pig sex	Strain	Phase	Guinea-pig weight	Test dose	Death in days	Loss in weight
♀	Low	I	318	63	2	—
♂	"	"	360	58	9	41
♀	"	"	371	57	10	37
♂	"	"	467	47	6	39
♂	H.W.	I	375	56	Lived	0
♂	"	"	396	54	12	40
♂	"	"	416	52	Lived	37
♂	"	"	434	50	6	38
♂	Parke Davis	III	346	60	5	34
♂	"	"	368	57	5	36
♂	"	"	398	54	5	30
♂	"	"	394	54	6	37
♂	H.W.	III	349	59	3	—
♀	"	"	369	57	10	43
♂	"	"	406	53	4	—
♀	"	"	490	46	11	49

Dose =  $\frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0.75}}$  expressed as nearest whole number.

\* 3½ months afterwards reinjected with Dose 50—death in 3 days.

† 3½ months afterwards reinjected with Dose 56—lived with 4% loss weight.

‡ Died during vaccination. P.M. cause of death unknown—cultures negative.

The general results of our immunity experiments therefore are irregular, and further work is clearly needed. But the evidence so far obtained, coupled with the serological findings, indicates that for prophylactic vaccination in the human subject Phase I vaccines made from recently isolated strains are more promising than those made from old cultures.

The work of Teissier, Reilly, Rivalier and Cambassédès (1929) on the immunising properties of *pertussis* endotoxin, which came to our notice after the completion of this work, shows that a more solid protection can be obtained with formolised endotoxin than with formolised bacilli. It also suggests that older cultures, presumably some of them in Phase III, are by no means devoid of endotoxin. Some experiments we have done on the effects of intradermal injections of cultures in rabbits have also led us to suspect this. These authors are pessimistic about the possibilities of immunising human beings,

owing to the intensity of the reactions and the apparent failure to produce antibodies; but there is no reason to believe that the last word on this subject has yet been said.

Our thanks are due to Dr Kristensen and Miss L. Mishulow for their kindness in helping us with cultures and other materials, and to Prof. Georges Dreyer for valuable criticism and advice. We should also like to acknowledge with gratitude the financial support we have received from the Medical Research Council.

ADDENDUM. Since this paper was written we have isolated 11 more strains of *H. pertussis*, all of which were in Phase I.

#### SUMMARY.

*Haemophilus pertussis* is a uniform species, without fixed varieties or "types."

After isolation from the human subject it tends to pass through a series of antigenically distinct phases, of which the first two are toxic to guinea-pigs, whereas the last two are relatively harmless. The former probably correspond to the smooth, the latter to the rough phase of other bacteria.

The phase-changes are, up to a point, reversible.

Experiments on guinea-pigs have produced some evidence that the toxic Phase I is the best and perhaps the only antigen for the production of active immunity in guinea-pigs.

#### REFERENCES.

- BORDET, J. and SLEESWYCK (1910). *Ann. Inst. Pasteur*, **24**, 476.  
 BORDET, J. (1913). *Centralbl. f. Bakt. u. Paras.* 1 Abt. Orig. **66**, 276.  
 DEBRÉ, R., MARIE, J. and PRETET, H. (1928). *C.R. Soc. Biol.* **98**, 761.  
 DREYER, G. and RAY, W. (1911). *Phil. Trans. Royal Soc. (London)*, B, **202**, 191.  
 FERRY, N. S. and NOBLE, A. (1918). *J. Bact.* **3**, 193.  
 KRISTENSEN, M. (1922). *Comm. Inst. Sérothérap. de l'État Danois*, **13**, No. 2.  
 — (1927). *C.R. Soc. Biol.* **96**, 355.  
 KRUMWIEDE, C., MISHULOW, L. and OLDENBUSCH, C. (1923). *J. Infect. Dis.* **32**, 22.  
 MEDICAL RESEARCH COUNCIL (1920). *Special Report Series*, No. 51.  
 TEISSIER, P., REILLY, J., RIVALIER, E. and CABBASSÉDÈS, H. (1929). *J. Physiol. et Path. Gén.* **27**, 549.

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# THE STUDY OF EPIDEMIC DISEASES AMONG WILD ANIMALS

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## I. INTRODUCTION.

FLUCTUATIONS in numbers of wild animals are of importance both in pure and applied ecology. The present paper is a contribution towards co-ordination of the scattered work that has been published on one phase of the main problem. Although a considerable amount of work has been done on epidemic diseases among wild animals, it has mostly consisted of sporadic and independent pieces of research. One of the objects of this paper is to show the value and importance, and in fact, the necessity of widely co-ordinated studies, in obtaining further advances in the subject. I am indebted to the Empire Marketing Board and the Hudson's Bay Company for supporting my research, during the course of which the data presented here were compiled. An appendix to this paper includes several notes upon epidemics observed, which have been communicated to me by naturalists. It was thought best to keep these separate from the summary of published data.

## II. THE OCCURRENCE OF DISEASE AMONG WILD ANIMALS.

Up to the present time it has been customary to believe that wild animals possess a high standard of health, which is rigidly maintained by the action of natural selection, and which serves as the general, though unattainable, ideal of bodily health for a highly diseased human civilisation. This belief is partly true and partly false. It is probably true that animals are normally much more healthy than human beings, and that the weaker individuals are weeded out by the enemies of the species and the occasional severity of outer conditions. At the same time, wild animals are not free from *epidemic* diseases, which are of very widespread occurrence among them. The systematic study of disease in wild animals forms one of the latest branches of animal ecology, and it is therefore impossible to make many generalisations which can be of

any permanent value; but it is safe to say that many species of wild animals suffer from outbreaks of disease no less severe than those which attack civilised man. In some cases (*e.g.* the Norwegian lemming) the decimating effects of these epidemics far exceed anything that is witnessed among human beings (Collett, 1911-12, p. 154). An important point to note is that, in a number of instances at any rate, it can be shown that the epidemics are not in any way attributable to human interference with the animals, or indirectly with their environment, nor contracted from domestic animals kept by men in artificial conditions. Disease is, in fact, a perfectly natural phenomenon, and it forms one of the commonest periodic checks upon the numbers of wild animals, especially in the case of mammals, being in this respect no less important than enemies, climate, food supply, and other generally recognised regulating factors. Such epidemic diseases are usually associated with overcrowding in the population, though they may not actually be caused by it directly. In consequence of this, there is usually a rather well-marked fluctuation in the numbers of the population, great density being followed by great scarcity, and this by a period of gradual increase up to another maximum, which is in turn followed by another epidemic. It appears that one of the chief reasons why so little attention has been paid in the past to the pathology of wild animals is the general and widespread belief of biologists that the numbers of wild animals remain constant, whereas in fact they fluctuate more or less violently from year to year. Fluctuations in numbers are also of course commonly caused by other ecological factors such as weather variations.

The importance from a medical point of view of studying epidemics in wild animals is fourfold. First, it is clear that our general attitude towards health in the human population must be affected, if it can be shown that epidemic disease is a normal and frequent phenomenon in nature. Secondly, the existing theories as to the origin of human disease in history may have to be reconsidered; for example, the idea that disease originated through the overcrowding caused by the town life of early civilisations. Thirdly, the widespread existence of a number of unstudied epidemic diseases in wild species with which man and his domestic animals are daily in contact directly or indirectly, offers a possibility of tracing some of the epidemic diseases of man and domestic animals to reservoirs in wild species. Finally, the pathologist is put in possession of a new means of studying epidemics in animals besides man, and thus is able to extend in a different direction the important work upon epidemics in cage-mice initiated in 1919 by Topley (Topley, 1919; Greenwood and Topley, 1925). Owing to the fortunate fact (which we shall discuss more fully in the next section) that these wild animal epidemics can frequently be forecast with some accuracy, it is possible to study the forerunners of an epidemic—a thing which it is very seldom possible to do properly in human outbreaks.

It will be seen, therefore, that there is at least the chance that some light might be thrown upon the mechanism of epidemics in general by a study of

the diseases of some wild animals. We must now consider briefly the distribution of disease among animals, as far as is known at present. Amongst invertebrates such as insects, climate and other factors than disease appear to play the chief part in producing sudden fluctuations in the population. But even here, periodic disease plays its part, as instanced by epidemics in the European crayfish, *Potamobius* (Brocchi, 1896), in the freshwater shrimp, *Gammarus pulex* (Goodrich, 1928), amongst locusts and grasshoppers (Uvarov, 1929) and of septicaemia among cutworms (*Euxoa*) (King and Atkinson, 1928). It is when we come to the vertebrates, however, that disease begins to loom importantly in the lives of animals. Natural epidemics have frequently been noted amongst freshwater fish (Fleming, 1871, 1, 150, 207, 228, 259; Williamson, 1929) and they occur also in various birds (especially game birds), e.g. diphtheria in wood pigeons, *Columba palumbus* (Scone, 1927); coccidiosis in willow grouse, *Lagopus lagopus*, in Norway (Brinkmann, 1926); and gapes (*Syngamus trachea*) in young rooks, *Corvus frugilegus* (Elton and Buckland, 1928); also epidemics among passenger pigeons (*Ectopistes migratorius*) in Ohio (Miner, 1929); house-sparrows and tree-sparrows (*Passer domesticus* and *P. montanus*) in the north of Scotland (Stenhouse, 1928; Inkster, 1929); and "plague" (a virus disease) of blackbirds in northern Italy (Todd, 1930).

Among wild mammals, epidemic disease is remarkably widespread in all parts of the world, occurring generally when the population has become unusually dense. The fluctuations in the population accompanying these epidemics are in some species so remarkable and have such far-reaching effects upon the other animals associated with them, and often also upon the economic, agricultural, and medical problems of man, that there is some very good evidence available on the subject. The lemmings (*Lemmus*, *Dicrostonyx*, *Myopus*) and mice (*Microtus*, *Pitymys*, *Apodemus*, etc.), in all parts of the world in which they have been studied, undergo violent fluctuations in numbers which are often associated with considerable epidemics. A typical example of a mouse plague followed by an epidemic on a large scale occurred in Nova Scotia (Canada) in 1815, when the early settlements were invaded from the surrounding forest by hordes of field-mice (Patterson, 1886). The increase occurred in a zone about 80 miles long and 50 miles broad (i.e. over an area of about 4000 square miles). By midsummer there were enormous swarms: cats, dogs, martens and foxes appeared also in numbers and gorged on the mice, and many cats became feral and multiplied excessively. Most of the hay and corn was destroyed. The mice disappeared rather quickly in the autumn and winter. They were seen crawling about slowly in a languid way, and then began to die in hundreds. Some of them attempted to migrate as well. Next season there was hardly a mouse in the whole district, except in one small locality where the abundance of mice continued for several years.

In Norway, the lemmings living up in the mountains and on the northern tundras increase every three or four years, and migration usually ensues on a large scale (Collett, 1911-12; Elton, 1924). The epidemic disease which kills



off the Norwegian lemming was stated by Horne to be due, in four outbreaks, to a specific bacillus (*B. pestis-lemmi*), said to be allied to the *Pasteurella* which causes avian cholera, and which invades the blood and organs in enormous numbers (Horne, 1912); while Collett states that a high proportion of the lemmings suffer from a skin disease which was found by Johan-Olsen to be associated with a species of bacterium which he named *Streptothrix lemani* (Collett, 1895). Doubtless other organisms also play a part in the tremendous decimation of the population which occurs after each epidemic. The lemming migrations in Norway occur every three or four years. Thus 1922 and 1926 were lemming years, and the next was expected and took place in 1930. Another animal which has been intensively studied of recent years is the South African gerbille (*Tatera lobengula*), a species of desert rodent, which appears to undergo fluctuations similar to those of the lemming, and with probably about the same period of three or four years (Pirie, 1927, p. 138). Its epidemics have attracted considerable attention from local authorities in the Union of South Africa, owing to the spread of the bacillus of bubonic plague (*B. pestis*) in that country during recent years. It seems probable that this rodent normally dies from diseases harmless to mankind (e.g. the Tiger River bacillus, *Listerella hepatolytica* (Pirie, 1927, p. 163)), but that bubonic plague, in its spread across the desert region, replaced these harmless organisms during the epidemics of 1923-4, with serious results to the human population, and to a lesser extent again in 1928, when epidemics due to a new species of *Pasteurella* were also recorded (Mitchell and others, 1930).

The Canadian snowshoe rabbit or varying hare (*Lepus americanus*), whose chief home is in the northern forest region of Canada, is subject to astonishing fluctuations in numbers, which are vividly reflected in the fur returns of the Hudson's Bay Company, owing to the fact that certain fur-bearing animals such as the lynx and red fox are mainly dependent upon the snowshoe rabbit for food. The cycle of the rabbit covers about ten years, and although the periodic abundance can be shown to depend partly upon climatic conditions, disease usually plays an important part when the animals become overcrowded (Elton, 1924). Describing the rabbit maximum of 1886 in Manitoba, Seton (1928) said: "The abundance that year was prodigious." During the following winter after the epidemic had broken out "in Manitoba, the country from Whitemouth to Whitesend, 250 miles long by 150 miles wide, was flecked with the bodies of white-furred Hares....The summer and fall of 1892, I spent in the same region, and did not see a single snowshoe." The epidemic just described appears to have been unusually great, but not without parallel, and local outbreaks occur in one part or another of Canada every ten years. It is of some interest to note that the snowshoe rabbit cycle has been going on without a break for at least 100 years, as shown by the fur returns and other records for the rabbits and the lynx.

In the western parts of the United States, similar epidemics occur amongst wild snowshoe rabbits and jack rabbits (Nelson, 1909; Palmer, 1896; Warren,

1910), and whereas we know nothing about the nature of the diseases which attack the Canadian snowshoe rabbit over an area of some millions of square miles, in the United States some light has been thrown on the matter by recent work. In certain cases rabbits have been found dying of tularaemia, a bacterial disease which is highly infectious to man (Green, 1928; Redington, 1928). Outbreaks of tularaemia in several parts of Russia have been recently traced to water-rats (*Arvicola*) (Roubakine, 1930), and the disease has also occurred in Norwegian hares, from which people have been infected (Thiðtta, 1930, 1931). In Eastern Siberia and Mongolia the steppe marmot or tarbagan (*Arctomys bobac*) is one of the most important carriers of plague, and the periodic epidemics to which it is subject have been proved by Wu Lien Teh (1924) to be one of the main foci of bubonic plague in those regions.

It would take too long to enumerate in detail all the cases of natural disease which are known now to occur among wild animals, and we therefore sum up the available information in the table given below. This table is not exhaustive, owing to the extremely scattered nature of the information, but it should be sufficient to indicate the great richness of the field which lies open for research upon the diseases of wild mammals.

Table I. *Fluctuations in numbers of wild animals, associated with outbreaks of disease.*

LEMMINGS (*Lemmus*, *Dicrostonyx*).

Fluctuations in numbers (four-year cycle, usually) in Norway (Elton, 1924, 1925). Horne (1912) studied the bacteriology of lemming epidemics in 1896, 1903, 1909 and 1910, and found *B. pestis-lemmi*, which was also fatal to guinea-pigs and other animals. Collett states that skin disease is frequently found in old individuals during lemming years. Specimens obtained by Collett in 1891 were examined bacteriologically by Johan-Olsen, who found a micro-organism ("*Streptothrix lemmi*") commonly associated with the skin lesions (Collett, 1895). An apparently similar skin affection found by C. Elton in old individuals of migrating lemmings in Finnmark, during the summer of 1930. Outbreaks of a kind of gastric fever (known as "lemming fever") in human beings, and apparently also in domestic animals, occur locally during and after lemming years in Norway (Collett, 1895). These outbreaks are attributed to infection of drinking water by dead or dying lemmings, or by their excreta. The subject has never been properly investigated, but the circumstantial evidence is convincing.

Similar fluctuations occur in the populations of lemmings in Sweden, Finland, Russia, Novaya Zemlya, Siberia, Alaska, Arctic Canada, Greenland, Baffin Island, and Labrador (general summary by Elton, 1924, 1925; Novaya Zemlya, Pearson, 1898; Western Siberia, Sedelnikov, 1907; Baffin Island, Kumlien, 1879).

Although epidemics probably play a part in these fluctuations, they have never been investigated.

VOLES OR FIELD-MICE (*Microtus*).

Violent fluctuations in numbers, often associated with epidemics, have been recorded from Great Britain, Norway, Germany, Austria, Czechoslovakia, Russia, Thessaly, Hungary, France, Italy, the Aegaeon Islands, Crete and Cyprus, Asia Minor, Palestine, North-eastern Siberia, Kamchatka, Alaska, the whole of Canada, Labrador, and many parts of the U.S.A. The references to the evidence on this subject are so numerous and scattered that they cannot be given here, and will be dealt with in a separate publication. It must be sufficient to refer to the text (pp. 443 *et seq.*) for the mouse cycles in Britain, Norway, and Bavaria. The evidence about epidemics in voles is discussed on p. 453.

FIELD-MICE (*Pitymys*).

Enormous mouse plagues in Italy, notably in 1916, when the mice died from epidemics of bacterial origin (Martelli, 1919).

RED-BACKED VOLES (*Evtomys*).

These mice, which are the typical forest-mice of the more northerly forests of both the Old and the New Worlds, have been shown to undergo important fluctuations in numbers

Table I (continued)

in several places, e.g. Ontario, Canada (Dymond, Snyder, and Logier, 1928, p. 243); Alaska (Sheldon, 1930, p. 121); and England (Pitt, 1918). It is not known if these fluctuations are accompanied by epidemics. A definite epidemic has been observed in Norway by Dr H. M. Blair (see Appendix, p. 453).

#### DEER-MICE (*Peromyscus*).

One of the commonest field- and forest-mice of the less northerly parts of North America, and occupying more or less the niche filled by *Apodemus* in the Old World. Fluctuations in numbers are frequent, and there are many records of sudden scarcity following great abundance. Dymond, Snyder, and Logier (1928, p. 244) say: "May it not be that the white-footed mouse (*Peromyscus*) plays a similar rôle in the northern coniferous forest of Canada to that played in the more northerly regions of Europe and America by lemmings and voles?" No direct evidence about epidemics has been recorded.

#### WOOD-MICE OR LONG-TAILED FIELD-MICE (*Apodemus*).

Took part in a great vole plague in the forest of Dean, England, in 1813 and 1814 (Douglas, 1825); mortality in the New Forest, England, in 1923, associated with similar deaths in voles (Longstaff, cited in Elton, 1924, p. 142). Spontaneous epidemic among voles and *Apodemus* at Charny, France, in February, 1893, from which Danysz obtained cultures for mouse-control work (Danysz, 1893); the numbers fluctuate greatly in Norway (Collett, 1912, p. 172). Frequently forms "plagues" of mice in French agricultural areas (Segnier, 1924) and took part in the vast mouse year that troubled Germany in 1918 (Schwarz, 1918), and Italy in 1916 (Martelli, 1919). On the Volga steppes *Apodemus*, among several other species, has been convicted as a permanent reservoir of bubonic plague, which bursts out in periodic epidemics (Dr I. Ioff, *in litt.*).

#### WILD MICE (*Mus*).

Australia. Periodic plagues in the wheat belt (both in the fields and in the bush), e.g. in 1916-17, when many mice died from a skin disease "somewhat resembling ulcerative syphilis" (Hinton, 1918), while the men who were handling the stacks contracted a skin disease on their arms, necks, and shoulders; the mouse plague was followed by a plague of fleas, which attacked human beings (information through Dr J. R. Baker).

Bubonic plague carriers on Volga steppes (Dr I. Ioff, *in litt.*).

#### GERBILLES (*Tatera*, *Desmodillus*) and other rodents.

Great fluctuations in numbers on the steppes and deserts of South Africa, associated with several different epidemic organisms (see text, p. 438).

#### GERBILLES (*Rhombomys*).

Epidemic of unknown nature in Kara Kum Desert, Turkestan, in 1927 (Kashkarov and Kurbatov, 1930).

#### HAMSTERS (*Cricetulus*).

Associated with outbreaks of bubonic plague in Mongolia (summary in Elton, 1925); took part in the great mouse year in Germany in 1918 (Schwarz, 1918).

#### COMMON RATS (*Mus*).

Associated with outbreaks of bubonic plague almost all over the world. In many areas plague periodicity is directly due to periodic epidemics among the rats themselves. A natural epidemic cycle of this sort appears to have existed in English rats in East Anglia between the years 1906 and 1918, and *Bacillus pestis* was proved to be causing deaths both in rats and human beings (literature briefly summarised in Elton, 1925).

#### WATER-VOLES (*Arvicola*).

Fluctuate in numbers, coincidentally with the lemmings and voles in Norway (Collett, 1912, p. 106; Wolley, 1856).

Proved to be the reservoir of outbreaks of tularaemia in Russia (Roubakine, 1930).

#### MUSKRATS (*Fiber*).

Marked cycle in numbers in various parts of Canada, partly associated with epidemics, e.g. of liver disease (MacFarlane, 1905, p. 737).

#### BEAVER (*Castor*).

Local epidemics in beaver colonies in the MacKenzie River region of Canada (MacFarlane, 1905, p. 743).

#### SQUIRRELS (*Sciurus*).

Marked fluctuations in numbers in Great Britain and Ireland (Middleton, 1930*b*); in Norway (Collett, 1912, p. 223, Wheelwright, 1871, p. 230); in Denmark (Hansen and Schiötte, 1892); in Siberia (Elton, 1925); and in Canada (Seton, 1920).

In the case of British squirrels Middleton (1930*b*) has obtained convincing evidence that the periods of decline in numbers are very often associated with disease, the nature of which is still uncertain.

Table I (*continued*)**MARMOTS (*Arctomys*).**

Proved to be one of the most important reservoirs of bubonic plague in Mongolia (Wu Lien-Teh, 1924). There is a good deal of evidence that the epidemics in marmots are associated with periods of abundance (Elton, 1925).

**GROUND SQUIRRELS (*Citellus*).**

Convicted as reservoir of bubonic plague and also of tularaemia in California (McCoy, 1910; Francis, 1925).

**SUSLIKS (*Citellus*).**

Periodic outbreaks of bubonic plague in north Caucasus steppes (Sveridenko, 1929).

**VARYING HARES OR SNOWSHOE RABBITS (*Lepus*).**

Very marked fluctuations in numbers over nearly the whole of their range, in Canada, Alaska, and also in Montana (Seton, 1920, p. 96; Seton, 1928; Elton, 1924; Sheldon, 1930, p. 329; Howell, 1923). Epidemics of unknown nature occur over many thousands of square miles, but have never been adequately investigated. Recently tularaemia has been found in Canada (Hudson, 1930), but the circumstantial evidence does not favour the idea of the widespread occurrence of this particular disease in Canada, although it is said to have been found several times among varying hares in the U.S.A. (Green, 1928).

**JACK RABBITS (*Lepus*).**

Subject to occasional epidemics on a large scale in many parts of their range; Western U.S.A. in general (Nelson, 1909), Colorado (Warren, 1910), California, Nevada, Idaho, Utah, and Washington (Palmer, 1896). Proved to be dying of tularaemia in Utah in 1919 (Redington, 1928).

**ALPINE HARES (*Lepus*).**

Periodic epidemics reported in England and Scotland, mostly of unknown cause, but in one case at least proved to be coccidiosis (Ritchie, 1926). Recently suspected of being reservoir of tularaemia in Norway (Thiðtta, 1930). Collett (1912) states that epidemics attack hares in Norway in certain years, causing disease of the lung, and associated with stronglylid worms (see also Appendix to this paper, p. 454).

**COTTON-TAIL RABBITS (*Sylvilagus*).**

Said to take part in epidemics with snowshoe or jack rabbits in U.S.A. (Nelson, 1909).

**COMMON RABBITS (*Oryctolagus*).**

Frequent epidemics in rabbit populations in the British Isles, little studied. References too numerous to summarise. Apparent causes in some cases are coccidiosis, liver fluke, and rabbit syphilis.

**WEASELS (*Mustela*).**

Probably subject to outbreaks of disease of unknown cause in Manitoba, Canada (Criddle, 1925).

**BADGERS (*Meles*).**

Subject to outbreaks of disease of unknown cause in Manitoba, Canada (Criddle, 1925).

**RED FOXES (*Vulpes*).**

In Britain they appear to suffer from periodic outbreaks of disease called "yellows" and believed to be spirochaetal jaundice (Dunkin, 1926). Certainly subject to outbreaks of nervous disease in various parts of Canada (Elton, through the Hudson's Bay Company, unpublished) and also in Kamchatka (Bergman, 1927).

**ARCTIC FOXES (*Vulpes*).**

Suffer from periodic outbreaks of a nervous disease resembling encephalitis, which is communicable and fatal to sledge-dogs, in the arctic parts of Canada. These outbreaks probably occur just after the periodic decline in numbers of lemmings (Elton, through the Hudson's Bay Company, unpublished).

**MOLES (*Talpa*).**

Occasional mortality in England, on a more or less large scale, as in 1926. The causes are unknown (see Appendix, p. 454).

**SHREWS (*Sorex*).**

Periodic fluctuations in Norway, often at times of lemming abundance (Collett, 1912, p. 9). In Britain they are subject to epidemics which kill them off in large numbers (*e.g.* in Denbighshire in 1925; Moffat, 1926).

**DEER (*Cervus*).**

Periodic epidemics in early years in Scotland (Fleming, 1871, p. 243).

**DEER (*Odocoileus*).**

Periodic cycle in numbers on Vancouver Island and in Western British Columbia not investigated for disease (Brooks, 1926).

Table I (*continued*)**ELK OR MOOSE (*Alces*).**

Epidemics due to unknown causes in Lithuania (Barnard, 1864) and Canada (Dymond, Snyder, and Logier, 1928, p. 248; Bradshaw, 1916).

**CHAMOIS (*Capreolus*).**

Epidemic of infectious disease causing blindness in Switzerland (Anon., 1927).

**WILD SHEEP (*Ovis*).**

Said to suffer from periodic epidemics every twenty years or so in the Pamirs (Cumberland, 1895).

**AFRICAN ZEBRAS.**

Periodic outbreaks of lungworm in East Africa (Percival, 1924).

**HIPPOPOTAMUS.**

Epidemics of unknown cause in the Congo (Hilton-Simpson, 1911; see Appendix to this paper, p. 453).

Only a few references have been given to plague (*Bacillus pestis*) in animals, since so many of the records are only isolated cases of infection, and not associated with epidemics on a large scale. A list of animals which have been found dying of bubonic plague is given by Wu Lien-Teh (1925), and criticised and amended by Pirie (1927, p. 122). A brief summary of some of the evidence associating plague epidemics with important fluctuations in the populations of wild animals is given by Elton (1925) and the recent work on this question in South Africa is summed up by Pirie (1927, p. 138), and Pirie and Murray (1927).

It will be observed from Table I that two of the diseases (bubonic plague and tularaemia) which produce epidemics among wild animals also produce epidemics in man. Our biological knowledge of both these diseases is very recent, since it was not until about the beginning of the twentieth century that plague was conclusively proved to arise from rat epidemics, and not until 1923 that it was finally proved to originate in marmots (although the connection was suggested by Clemow in 1900, on a basis of strong circumstantial evidence). Tularaemia is a still more recent discovery, and dates from 1911 and 1912.

It is obvious that the pathology of wild animals is a subject that has hardly passed beyond the observational stage as yet, and that the unknown elements must be incomparably more numerous than the known. The comparative success of medical work in regard to man, both on the bacteriological and pathological sides, has made it possible to forget that our knowledge of disease in wild animals is very meagre. It would hardly be possible, for instance, to sum up our present knowledge of human epidemics in a short table such as is given above! In view of the increasing speed and perfection of modern transport, there are growing up new opportunities for the spread of diseases capable of finding fresh reservoirs in wild animal populations.

## III. FORECASTING EPIDEMICS AMONG WILD RODENTS.

The table given in the previous section (p. 439) shows that epidemic diseases are not uncommon amongst wild animals, and that very little is known about their nature or their causes. One reason for this state of affairs has been the difficulty of knowing when to expect the outbreaks. For instance, in the early days of plague research in China, it was for some years almost impossible to prove conclusively that the Mongolian marmot (*Arctomys bobac*) was the actual reservoir of plague for Chinese outbreaks, because scientific expeditions to the districts where the marmots were dying usually arrived after the epidemic had run its course. As a result of this it remained for some years doubtful whether the marmot outbreaks arose from the human plague epidemics or *vice versa* (Wu Lien-Teh, 1922). Finally the establishment of a permanent station to watch for the occurrence of disease, made it possible to demonstrate conclusively the connection between marmot epidemics and human outbreaks of plague. But it proved necessary to watch for about ten years before success was achieved (Wu Lien-Teh, 1924).

The same difficulty is encountered in the study of any other animal epidemics, and is, of course, a prime cause of our ignorance of the mechanism of epidemic diseases in human beings. It is obvious that a reasonably accurate forecasting system for epidemics would enable research to be focussed more efficiently on the problem and would make it possible to go to the right place at the critical moment, and thus avoid the uncertainty, and the waste of time and money which otherwise occur.

Now, the remarkable fluctuations in the population which usually accompany epidemics in certain wild animals, make the building up of such a forecasting system quite feasible, provided enough information can be obtained about the changes in numbers from year to year, and about the course which the cycles have run in the past.

The numbers of mice and lemmings are known to fluctuate in all parts of the world where the subject has been investigated; in Labrador, in California, in the wheat-belt of Australia, in the deserts and steppes of South Africa, on the mountains and also on the lowlands of Scandinavia, on the Volga steppes, over most of central Europe, and in Great Britain. The earlier records that we possess refer simply to "mice," without giving details about the species concerned, and they are mainly descriptions of very large "plagues" of mice, during which crops were eaten up and destroyed. Several such "plagues" are mentioned in the Bible, and they are noticed fairly often in the records of Greek and Roman history. Such large plagues occurred in Alsace in the years 1271, 1278, 1366, 1378, 1451, 1468, 1479, 1501, 1538, 1593, 1617, 1652, 1685, 1719, 1794, 1801-2, 1818, 1822, 1856, 1861 (Gerard, 1871). This list gives an idea of the sort of periodicity shown by really great "mouse plagues," and that there is no especial regularity in their occurrence. (In this case there are 21 in 600 years.) If we examine the records for some country over the

last hundred years we find that there are, in between the great mouse plagues, a good many smaller periods of unusual increase which would not, however, have been sufficiently important to become preserved in the earlier historical records. Thus, it is recorded that the department of the Aisne in France suffered from unusual increase of field-mice in the years 1865, 1870 and 1880 (Chavée-Leroy, 1882), 1909, 1912 and 1919 (Segnier, 1912; Marchal, 1919). It will be seen that the "mouse years" in these two series were three to ten years apart.

This fact leads us to enquire whether there may not be still smaller "mouse years" in which the population reaches a relatively large peak in numbers, which is not absolutely large enough to attract attention through the economic damage which it would cause if the mice were more numerous. In order to discover whether this is so, that is to say whether mouse plagues, in the economic sense, are only unusually big periodic maxima in a natural cycle of numbers, it is necessary to have special means of investigating the periodicity.

It is not easy to carry out direct censuses of wild mice, and such attempts have only been made within the last few years. For obtaining data over a long period of years we have therefore to rely upon indirect means of detecting variations in the numbers. So far, these methods have been applied with success four times; in the study of lemmings and mice in Norway, lemmings in Canada, mice in Great Britain, and mice in Bavaria.

1. **LEMMINGS AND MICE IN NORWAY.** Lemmings (*Lemmus*, *Dicrostonyx*) are northern representatives of the vole family, whose members are the ecological equivalents of the short-tailed field-mice (*Microtus*, etc.) of the temperate regions. As far as the present problem is concerned, mice and lemmings may be treated as one unit. Owing to the fact that there is a backbone of mountain running from north to south down the centre of Norway, the arctic-alpine zone of vegetation extends far south of the arctic circle, and covers large areas of the Dovre, Jotunheim, and other mountain plateaux of southern Norway, and is inhabited by the Norwegian lemming (*Lemmus lemmus*), which also occurs on the lowland tundras much farther north. The lemming population is subject to the same fluctuations which occur among field-mice, and owing to the peculiar position of the lemmings in southern Norway, and the great amplitude of their fluctuations, they frequently overflow their natural habitat and visit the lowlands. These migrations have long been a subject of wonder and comment, and present many interesting features of their own; but the point with which we are concerned here is that they give an index of the periods of over-population in the lemming habitats. The dates of these migrations have been recorded by Collett (1912) and others, and the periodicity has been studied by Elton (1924). It is found that migrations occur in some part of central or southern Norway at very regular short intervals of three or four years; but they do not necessarily occur over the whole of this region, as we should expect if migration is only an index of unusually great over-population. In the period between 1860 and 1930 this periodicity in migrations (and there-

fore in numbers) has been maintained, and in the course of these seventy years there have been only two occasions (1897 and 1914) when the records do not show a migration at the time expected. These two exceptions were most certainly cases when over-population was not sufficiently great to cause migration, since all degrees of migration have been noted, from enormous waves of lemmings marching in thousands and reaching the sea-coast (*e.g.* 1909 and 1926), to small local migrations such as those recorded for 1880 and 1918.

We conclude from this evidence that there is a regular cycle in numbers of lemmings on the mountains of central and southern Norway, with a periodicity of three or four years. As mentioned on p. 439, epidemics play an important part in the phase of decrease in numbers. A number of parallel records, of a less complete nature, prove that the numbers of wild mice on the mountain slopes and foothills and lowlands usually reach a peak in the same years as the lemming. Furthermore statistics of the government bounties paid for arctic fox and red fox and birds of prey, show peak years at regular intervals, and these correspond with the lemming cycle, and show maxima after 1897 and 1914 when there were no actual migrations of lemmings recorded (Johnsen, 1929). On the basis of this periodicity I forecasted that 1930 would be a lemming year in Norway and ventured a visit on this prediction. It was in fact an important lemming year over the whole of Norway.

2. **LEMMINGS IN CANADA.** The lemmings are one of the chief foods of the arctic fox in the northern regions of Canada, and it can be shown that the numbers of the latter are probably an index of the numbers of the former. The arctic fox fluctuates in numbers very violently, and these fluctuations are shown in the fur returns of the Hudson's Bay Company (see Elton, 1924, 1925). This curve, which represents mainly the skin collection from Ungava Bay on the northern coast of Labrador, has a periodicity of three or four years. We conclude therefore that the Canadian lemmings have a cycle in numbers similar to that of the Norwegian lemmings—a conclusion which is supported by data about the lemmings themselves. A further striking fact is that the peak years for lemmings in Canada show some correlation with those in Norway, indicating that there may be a common climatic factor influencing lemmings in both countries.

3. **FIELD-MICE IN GREAT BRITAIN.** Most of the observations which have been made upon fluctuations in numbers of British mice refer to the short-tailed field-mouse (*Microtus*), which occurs all over England, Wales and Scotland, but is absent from Ireland. Long-tailed field-mice (*Apodemus*) may also take part in these fluctuations. An extensive investigation has been carried out by A. D. Middleton under the auspices of the Empire Marketing Board. This work has been published elsewhere (Middleton, 1930 and 1931), and will only be briefly summarised here.

By means of questionnaires and other sources of information from a number of people, it has been ascertained that local mouse plagues or "mouse



years" in Great Britain occur periodically, and that the periodicity is three or four years. Thus 1898, 1902, 1906, 1909-10, 1913-14, 1918, 1922, 1926 and 1929-30 were local mouse years. There is a very strong tendency for these mouse peak years to occur simultaneously in different districts. Thus there were mouse years in 1926 in Somerset, Westmorland, Cumberland, Roxburgh and Argyll. The simultaneous occurrence of these outbursts of numbers, and the comparative scarcity of mice over wide areas in the intervals between them, points to the existence of some controlling factor in climate. This idea is strongly supported by the remarkable correspondence of mouse years in Great Britain with mouse and lemming years in Norway. The tendency for synchronous and parallel fluctuation to appear in the numbers of small rodents on both sides of the North Sea, and the tendency for correlation between lemming years in Canada and Norway, may be taken to indicate some general climatic pulsation acting over the whole of that part of the North Atlantic.

4. **FIELD-MICE IN BAVARIA.** A great deal of work has been done upon the incidence and nature of "mouse plagues" in France and Germany, and numerous attempts have been made to control these outbreaks by means of various destructive measures. Much work has been done especially upon the introduction of various "viruses" (mostly cultures of bacteria belonging to the *Salmonella* group; see White, 1929), in order to initiate epidemics among the mice. This work has had a certain local success, but the effects of it have been usually masked and falsified by the fact that the mice have a natural cycle of their own which causes them to disappear after they become abundant. The existence of a regular and natural periodicity in numbers of field-mice was first pointed out by Hiltner (1916), and for a number of years he collected statistics which show conclusively that in Bavaria there is a very definite cycle in numbers, which varies somewhat from one region to another.

The forecasting of the mouse numbers in Great Britain is based therefore at present upon the following knowledge:

(a) Knowledge of the widespread and well-authenticated existence of cycles in numbers of small rodents, in many different countries.

(b) Knowledge that there is a fairly regular fluctuation in numbers of mice, probably controlled in some way by climate, which occurs more or less simultaneously in England and Norway and even in Canada.

(c) Past records showing that the interval between maxima has been usually four years, sometimes three, and rarely more or less.

(d) Information as to the date of the last maximum. Thus in 1925 we were aware that 1922 had been a mouse year in Hampshire (New Forest) and Somerset, also that 1922 was the year of a great lemming migration in Norway. The next maximum year would therefore be expected to occur in 1926 or possibly in 1925. An intensive piece of ecological work was started on this prediction, the results of which are being published elsewhere (Elton, Ford, Baker and Gardner, 1931).

(e) Information as to the actual state of mouse numbers at the time.

Thus, the comparative scarcity of mice when operations were started in the autumn of 1925, made it seem probable that the peak would not be reached until 1926. Negative information like this, however, is not really trustworthy, since in some peak years the mice may reach only relatively very small density of numbers. For instance it appears probable that the exceptionally severe frosts of the winter of 1928-9 greatly reduced the numbers of wild mice in some parts of England, so that the usual peak is beginning to show itself mainly in areas which had been protected by a snow covering, *e.g.* mountains of Wales and parts of Scotland. It is hoped, as a result of work which is going on at Oxford upon mice, to discover the exact manner in which mouse increase is affected by climate and thus make forecasting more accurate.

We may now turn to the important question of the relation of disease to cycles in numbers of mice, and the evidence that epidemics occur among wild mice at all. The periodic epidemics proved to occur among Norwegian lemmings and South African gerbilles have already been commented upon, while references are given in Table I, pp. 439, 440, to epidemics in many other small rodents such as hamsters, rats, etc. There is an enormous literature devoted to the study of vole plagues in Europe, dealing more especially with the means of controlling outbreaks by using poisons, bacterial diseases, and other measures. The more purely biological aspects of the problem that vole plagues present have not, however, been adequately studied, with the result that much of the work done by bacteriologists upon the control of mouse numbers by disease has been uncritical, based upon erroneous preconceptions, and, above all, lacking in adequate controls in the field experiments. The commonest type of experiment has been to introduce into the mouse population (then at or near its maximum abundance) cultures of *Salmonella enteriditis* or other strains of "mouse typhoid," and watch the effect upon the numbers of the mice. Usually successful results have been claimed. But it has often been noted by independent observers that the voles were disappearing in other areas where no control measures had been applied. In other words the experimenters have seldom reckoned with the fact that the mouse populations into which the epidemic cultures were introduced were probably nearly always at the point of generating some form of epidemic themselves. The recognition of the existence of natural epidemics among wild mice makes it extremely difficult to prove the efficacy of an artificially introduced disease at all, since the time of greatest abundance is the very time when both artificial and natural epidemics might be expected to begin successfully.

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The general truth of this situation has been admitted by more than one worker. Thus Rabaté (1914) said, "En effet, les grandes invasions disparaissent subitement, pour des causes encore mal connues, comme en novembre 1905.... La disparition générale semble due à une affection microbienne, épidémique, à propagation rapide, et intéressante à suivre de près, mais pour les campagnols rien encore sur ce point n'a été précisé." His remarks were made in

1914 at the time of a severe vole plague in France, which had been continuing for over a year unabated, in spite of all repressive measures.

A curious point about the work that has been done upon mouse-plague control by means of artificially introduced diseases, is the almost complete absence of published evidence that wild mice and voles ever die of mouse-typhoid epidemics, except when the bacteria have been deliberately introduced. With the exception of the original epidemic in February, 1893, from which Danysz (1893) first obtained cultures of "mouse typhoid," and several epidemics among cage-mice such as that recorded by Loeffler (1892), records on the subject seem to be very rare. At the same time it appears to be widely believed that mouse typhoid is a common source of epidemics among wild mice, and this belief has deflected investigation from the natural epidemics themselves. It would appear, on the contrary, that little is known about the actual organisms responsible for killing off *Microtus* at times of abundance, and that the belief in the widespread nature of mouse-typhoid epidemics has arisen mainly from the propaganda of vested interests concerned in the manufacture of control bacterial cultures on a large scale. It is significant that there is at the present time a general reaction against the use of such cultures, because the epidemics initiated by them are insignificant in extent and duration. The evidence on this point has been conveniently and impartially summed up by White (1929), while it may be noted that the official recommendations of the American Bureau of Biological Survey for mouse destruction do not mention bacterial cultures at all, but confine themselves to poisons and trapping (Silver, 1924). It seems fairly certain that this generally adverse opinion is not due merely to successful propaganda on the part of firms selling poisons for the same purpose! The importance of this point, which has been discussed at some length, is that if the disappearance of field-mice after "mouse plagues" is not caused by mouse typhoid, it must be caused by something else. Moreover, the cause of the mortality must be of a rather obscure nature, to have escaped the attention of the many bacteriologists engaged on a study of the problem. The facts at present known would be explained if we assumed that the voles die off periodically from some virus disease which leaves little trace on the tissues (as might be the case with a rapidly acting disease); and if at the same time we suppose that the epidemic is preceded by a period of lowered resistance to invasion (due for instance to food shortage or to the scarcity of some factor in the food). At such times we might expect the mice to be susceptible to some extent to other organisms, thus accounting for the definite though limited effects of the introduction of mouse typhoid. It should be remembered also that adequate technique for studying filterable viruses is only of recent origin.

Most of the evidence for epidemics in *Microtus* is circumstantial and consists of records of enormous multiplication in numbers, followed by very sudden disappearance, coupled with evidence that the voles have not simply migrated, and often with the discovery of numerous dead bodies which have

no signs of external injury. Innumerable examples of sudden and unaccountable disappearance could be quoted in connection with the European mouse plagues. A good example from Canada has already been described (p. 437). Preble (1908, pp. 186-8) described a mouse plague which overran central Saskatchewan and Alberta (the grain provinces of Canada) in 1900, and he said "immense numbers, many of which were floating down the rivers, were found dead." During the months of April in the following year (1901) he found fairly recent traces almost everywhere he went, between Edmonton and Fort Chipewyan, but practically no signs of any living mice. In 1907 there was a great mouse plague in Nevada, Utah, and north-western California. By November, 1907, the fields were honeycombed with the burrows of the mice, and much damage was done by them. By next August the voles had almost completely disappeared. Piper (1908, p. 302) said: "while the Nevada plague is the most serious recorded in the United States, frequent milder outbreaks in many parts of the country indicate that practically all our species of short-tailed mice periodically tend towards enormous multiplication." At intervals between January and March, 1908, dead and dying mice were noticed (the deaths being found not to be due to poison), but attempts to prove the mortality due to some specific bacterial disease were unsuccessful. Another great outbreak of mice took place in Kern County, California, during 1926-7; this time the main agent in the multiplication was the house-mouse (*Mus musculus*), which overran a newly planted area of land, and subsequently migrated for some distance into surrounding country. This was not, therefore, strictly comparable with the normal type of fluctuation in numbers of field-mice, but it is mentioned here both because *Microtus* also took part in the increase (although in smaller numbers) and also because both kinds of mice were later found to be dying from an epidemic of a disease which was studied by Wayson (1927) and found to be caused by *B. marsepticus*, an organism almost if not quite indistinguishable from that responsible for erysipelas in swine and human beings.

In England, through lack of attention, and also through the lesser economic importance of field-mouse plagues, there is not a very large amount of evidence about epidemics in voles. An epidemic among short-tailed and long-tailed field-mice was noted by Longstaff in the New Forest in 1923 (cited by Elton, 1924), and another by A. Moffat during the great vole plague in Scotland in 1892: "the voles seemed to get into a dormant state as if they were stricken by some disease...they disappeared underground, to be seen no more, where only a few days previously they had been running in thousands" (Moffat, cited by Middleton, 1930, p. 161). Several definite epidemics have been recorded from Scandinavia. Thus Wolley (1856) noted that three species of voles (*Microtus amphibius*, *agrestis* and *ratticeps*) increased at the same time as the lemmings in 1853 in northern Lapland (Muonioniska). During the spring of 1854 they nearly all disappeared, and dead and dying bodies were found, with no external injuries, very abundantly in hayricks, etc. An epidemic among

*Evotomys* in Norway, observed by Dr Blair, is described in the Appendix, p. 453.

It will be noted that most of these descriptions agree in one respect: the absence of any obvious symptoms of the type one has been led to expect in bacterial diseases. Plague and erysipelas are exceptions, but it does not appear that *Microtus* is a common carrier of these diseases; on the other hand the records of epidemics in other kinds of voles and mice are more specific. These facts tend to confirm the hypothesis that *Microtus* usually dies from some disease or diseases that will require a special technique for their examination.

The account which has just been given of mouse cycles and the research which is being developed in order to forecast their periods of abundance and scarcity, and their epidemics, illustrates a technique which can be applied to a number of other species about which at present we have much less knowledge. It can undoubtedly be applied to the ten-year cycle in Canadian wild animals such as the varying hare (snowshoe rabbit) and the ruffed grouse, and probably the muskrat. A full account of investigations which I have been carrying out in co-operation with the Hudson's Bay Company upon cycles in Canadian wild life, will be published in the early future. It is clear that ecology, and particularly that branch of it which deals with animal numbers, has a definite contribution to make towards the epidemiology of mammals, and also towards the solution of certain important problems in human disease.

#### SUMMARY.

1. Outbreaks of epidemic disease are common in populations of wild animals, including species little influenced by contact with the diseases of human beings or domestic animals.

2. Such epidemics form one of the commonest factors responsible for fluctuations in numbers of wild mammals.

3. An attempt is made to summarise the available published records of such epidemics (Section II), while certain unpublished records (communicated to me by naturalists) are contained in an Appendix.

4. Little is known of the causes of these epidemics except in the cases of plague and tularaemia.

5. The fluctuations in numbers of some wild mammal populations are sufficiently regular to make the forecasting of epidemics possible. This method is already applicable to wild mice.

6. Mouse periodicities are discussed in detail, with special reference to epidemics and their causes, which are mainly obscure (Section III).

7. Development of the forecasting methods described will make possible the prediction of many other wild mammal epidemics, and render intensive pathological and epidemiological studies more practicable than they have hitherto been.

## REFERENCES.

- ANON. (1927). *The Times*, July 30th.
- BARNARD, M. R. (1864). *Sport in Norway* (London), p. 147.
- BERGMAN, S. (1927). *Through Kamchatka by Dog-Sled and Skis* (London), p. 128.
- BRADSHAW, F. (1916). *Rept. of Chief Game Guardian*, Dept. of Agric., Province of Saskatchewan, Canada, pp. 21-5.
- BRINKMANN, A. (1926). *Bergens Museums Aarbok*, p. 3.
- BROCCI, P. M. (1896). *La Pisciculture dans les Eaux Douces* (Paris), p. 296.
- BROOKS, A. (1926). *J. Mammalogy*, **7**, 37.
- CHAVÉE-LEROY (1882). *J. Agric. Pratique*, **1**, 13.
- CLEMOW, F. G. (1900). *J. Trop. Med.* **2**, 169.
- COLLETT, R. (1895). *Christiania Vidensk.-Selsk. Forhandl.* No. 3, 52.
- (1911-12). *Norges Pattedyr* (Christiania). (In Norwegian.)
- CRIDDLE, N. and S. (1925). *Canad. Field-Nat.* pp. 142-8.
- CUMBERLAND, C. S. (1895). *Sport on the Pamirs and Turkestan Steppes* (London), p. 82.
- DANYSZ, J. (1893). *J. Agric. Pratique*, **2**, 920.
- DOUGLAS, S. (1825). *Zool. J.* **1**, 433.
- DUNKIN, G. W. (1926). *Vet. J.* **82**, 147.
- DYMOND, J. R., SNYDER, L. L. and LOGIER, E. B. S. (1928). *Trans. Roy. Canad. Inst.* **16**, 233.
- ELTON, C. S. (1924). *Brit. J. Exp. Biol.* **2**, 119.
- (1925). *J. Hygiene*, **24**, 138.
- ELTON, C. S. and BUCKLAND, F. (1928). *Parasitology*, **20**, 448.
- ELTON, C., FORD, E. B., BAKER, J. R. and GARDNER, A. D. (1931). The Health and Parasites of a Wild Mouse Population. *Proc. Zool. Soc. London*. (In the press.)
- FLEMING, G. (1871). *Animal Plagues* (London).
- FRANCIS, E. (1925). *J. Amer. Med. Ass.* **84**, 1243.
- GERARD, C. (1871). *Essai d'une Faune Historique des Mammifères Sauvages de l'Alsace* (Colmar).
- GOODRICH, H. P. (1928). *Quart. J. Micr. Sci.* **72**, 325.
- GREEN, R. G. (1928). *Bull. Amer. Game Protective Ass.* Aug.-Sept. p. 79.
- GREENWOOD, M. and TOPLEY, W. W. C. (1925). *J. Hygiene*, **24**, 45.
- HANSEN, H. J. and SCHIÖDTE, I. C. (1878-92). *Zoologica Danica*, **1**, Pattedyr, p. 88 (Copenhagen).
- HILTNER, L. (1916). *Praktische Blätter für Pflanzenbau und Pflanzenschutz*, **14**, 137.
- HILTON-SIMPSON, F. W. (1911). *Land and Peoples of the Kasai* (London).
- HINTON, M. A. C. (1918). *British Museum (Natural History) Economic Series*, No. 8, 44.
- HORNE, H. (1912). *Centralbl. Bakt. Parasit. Infekt.* **66**, 169.
- HOWELL, A. B. (1923). *J. Mammalogy*, **4**, 149.
- HUDSON, H. D. L. (1930). *J. Canad. Med. Ass.* May, p. 678.
- INKSTER, C. J. (1929). *Scottish Nat.* p. 10.
- JOHNSEN, S. (1929). *Bergens Museums Aarbok*, p. 113.
- KASHKAROV, D. and KURBATOV, V. (1930). *Ecology*, **11**, 49.
- KING, K. M. and ATKINSON, N. J. (1928). *Ann. Ent. Soc. of America*, **21**, 167.
- KOFOID, C. A. and CHRISTIANSEN, E. B. (1915). *Univ. Calif. Pub. Zool.* **16**, 23.
- KUMLIEN, L. (1879). *Bull. U.S. Nat. Mus.* No. 15, 53.
- LOEFFLER, F. (1892). *Centralbl. Bakt. Parasit. Infekt.* **11**, 129.
- MAOFARLANE, R. (1905). *Proc. U.S. Nat. Mus.* **28**, 673.
- MARCHAL, P. (1919). *J. Agric. Pratique*, **32**, 876.
- MARTELLI, G. (1919). *Contributo alla conoscenza della vita e dei costumi delle Arvicole in Puglia* (Portici).

- McCoy, G. W. (1910). *J. Hygiene*, **10**, 589.
- MIDDLETON, A. D. (1930 a). *J. Ecology*, **18**, 156.
- (1930 b). *Proc. Zool. Soc. London*, p. 809.
- (1931). *J. Ecology*, **19**, 190.
- MINER, J. (1929). *Rod and Gun and Canad. Silver Fox News*, Jan. p. 612.
- MITCHELL and others (1930). *J. Hygiene*, **29**, 394.
- MOFFAT, C. B. (1926). *Irish Nat. J.* **1**, 66.
- NELSON, E. W. (1909). *North American Fauna*, No. 29 (Pub. U.S. Bureau of Biological Survey, Washington).
- PALMER, T. S. (1896). *Bull. U.S. Dept. Agric.* (Div. Ornith. and Mammal.), No. 8.
- PATTERSON, G. (1886). *Canad. Record of Sci.* **2**, 472.
- PEARSON, H. J. (1898). *Ibis*, p. 194.
- PERCIVAL, A. B. (1924). *A Game Ranger's Notebook* (London), p. 303.
- PIPER, S. E. (1908). *U.S. Yearbook Agric.* p. 301.
- PIRIE, H. (1927). *Pub. S. Afr. Inst. Med. Res.* **3**, 138, 163.
- PIRIE, H. and MURRAY, W. A. (1927). *J. Med. Ass. S. Afr.* **1**, 12.
- PITT, F. (1918). *The Field*, Jan. 26th, 143.
- PREBLE, E. A. (1908). *North American Fauna*, No. 27 (Pub. U.S. Bureau of Biological Survey, Washington), p. 200.
- RABATÉ, E. (1914). *J. Agric. Pratique*, **27**, 47.
- REDINGTON, P. C. (1928). *Rod and Gun and Canad. Silver Fox News*, Aug. p. 216.
- RITCHIE, J. (1926). *Trans. and Proc. Perthshire Soc. Nat. Sci.* **8**, 156.
- ROUBAKINE, A. (1930). *Monthly Epid. Rept. League of Nations*, R.E. No. 134.
- SCHWARZ, M. (1918). *Mitt. Deutsch. Landwirtsch. Gesellsch.* **33**, 418.
- SCONE (1927). *British Birds*, **21**, 19.
- SEDELNIKOV, A. B. (1907). *Russia: Full Geographical Description* (edited by V. P. Semenov-Tyan-Shansky), **16** (West Siberia), 130.
- SEGNIER, H. (1924). *J. Agric. Pratique*, **41**, 126.
- SETON, E. T. (1920). *The Arctic Prairies* (London).
- (1928). *Lives of North American Game Animals*.
- SHELDON, C. (1930). *The Wilderness of Denali* (London).
- SILVER, J. (1924). *U.S. Dept. Agric. Farmers' Bull.* No. 1397.
- STENHOUSE, J. H. (1928). *Scottish Nat.* p. 162.
- SVERIDENKO, P. A. (1929). *Berichte des Mikrobiol. Staats-Inst. zu Rostow am Don*, No. 9, 162.
- THIÖTTA, T. (1930). *Nordisk Med. Tidskr.* March 22nd, p. 177.
- TODD, C. (1930). *A System of Bacteriology in Relation to Medicine*, **7**, 231.
- TOPLEY, W. W. C. (1919). *Lancet*, ii, 1.
- UVAROV, B. P. (1929). *Locusts and Grasshoppers* (London), p. 136.
- WARREN, E. R. (1910). *The Mammals of Colorado* (London), p. 36.
- WAYSON, N. E. (1927). *Pub. Health Rept. U.S. Pub. Health Service*, **42**, 1489.
- WHEELWRIGHT, H. W. (1871). *A Spring and Summer in Lapland* (London).
- WHITE, P. B. (1929). *A System of Bacteriology in Relation to Medicine*, **4**, 132-3.
- WILLIAMSON, I. J. F. (1929). *Fishery Board for Scotland, Salmon Fisheries*, No. 1. (See also summary in *Lancet*, 1930, ii, 305-6.)
- WOLLEY, J. (1856). *Forhandl. Skand. Naturforsk. Syvende Mode* (Christiania), p. 216.
- WU LIEN-TEH (1922). *J. Hygiene*, **21**, 62.
- (1924). *Ibid.* **22**, 329.
- (1925). *Proc. 6th Biennial Conf. Far Eastern Ass. Trop. Med.* (Tokyo).

## APPENDIX.

## SOME UNPUBLISHED DATA ON ANIMAL EPIDEMICS.

The following data have been communicated to me by naturalists during the course of my enquiries. I wish to thank them for permission to publish their first-hand observations here.

1. EPIDEMIC AMONG HIPPOPOTAMI IN THE CONGO. In 1911 Captain F. W. Hilton-Simpson published a note on this subject in his book *Land and Peoples of the Kasai* (p. 29), in which he stated: "During our journey up the Kasai, the captain of the *Velde* told us that about the year 1904 or 1905 a very deadly epidemic had broken out among the hippopotami of that river and the Sankuru. So great had been the mortality among the animals (which even now exist in the middle Kasai in almost as great numbers as in Wissman Pool) that the factories on the bank had been obliged to employ men with canoes to push out into the current the carcasses which had lodged on the shore close at hand, the stench from which, as they began to decay, had been appalling. I could gather no information as to the nature of this disease."

Captain Hilton-Simpson wrote to me as follows: "It is, however, interesting to note that whereas hippos were very common in the Kasai and Sankuru Rivers in 1907-9, my friend, Captain Douglas Fraser, who was out there last year (1927), could scarcely believe me when I told him that they were no extreme rarities. Neither he nor I believe that any human agency has exterminated them, and, on my mentioning the epidemic I refer to, he agreed with me that there have probably been one or two recurrences of it."

2. EPIDEMIC AMONG RED-BACKED VOLES (*EVOTOMYS RUFOCANUS*) IN NORWAY. During the years 1924-8 Dr H. M. Blair collected a great deal of valuable information about the fluctuations in numbers of wild mice in the north of Norway (Varanger Fjord). He has kindly allowed me to record here an epidemic observed among red-backed voles, *Evotomys rufocanus*, during the early summer of 1927. Voles had increased progressively during the years 1924-6 and "1926 was noteworthy as a mouse year; both species (*Microtus ratticeps* and *E. rufocanus*) literally swarmed around Svanvik in the Pasvik Valley, and in Nord-Varanger they were very abundant. The winter of 1926-7 was long and severe, the spring of the latter year very cold and backward, yet voles were as common as in 1926....In the early part of June 1926 many dead voles (*E. rufocanus*) were lying amongst the short grass of a little meadow surrounding my lodgings at Svanvik on the Pasvik River. In many cases no obvious cause of death could be discovered, but quite a number of the bodies showed a rather remarkable condition. In these large abscesses had formed either in the abdominal wall, or in connection with the external genitals. These collections of pus did not appear to be due to an actual injury with septic infection, but rather to a pyaemia. In the surrounding marsh no dead voles were found, but they would be easily concealed from sight by the thick growth of dwarf birch, *Ledum*, etc."



This record is important in three ways particularly. It was very fortunate that the epidemic should have been observed by a trained medical man; it is the only record of a definite epidemic in this genus *Evotomys* that I have heard of; and it can have had no connection with lemming epidemics since no lemmings visited Varanger Fjord in these years. Dr Blair adds: "In 1928, Dr Stewart of Ellera Bank tells me he did not see any signs of mice around Svanvik."

### 3. EPIDEMICS AMONG HARES (*LEPUS TIMIDUS*) IN THE NORTH OF NORWAY.

It has long been known that hares in Norway are subject to outbreaks of disease (Collett, 1911-12, p. 64). In 1930 Thiðtta drew attention to the existence of tularaemia in human beings in Norway derived from hares, and this has focussed interest on the natural epidemics occurring among these animals, which have not been properly investigated. While working on rodents, during the Oxford University Lapland Expedition in 1930, I obtained three pieces of information about hare epidemics which seem worth putting on record.

(a) Mr Tharald Frette, Norwegian forestry officer at Karasjok (a Lapp settlement in the middle of Finmark, north Norway), informed me of the great fluctuations in numbers of hares noticed by him during the period he had been in the district. This information was obtained through the help of Mr Einar Mathisen, who was the Norwegian member of the expedition. Between 1920 and 1926 there were numerous hares every year. In 1926 there were still a great many, but fewer than in 1925. In 1927 the numbers began to drop heavily, and there were outbreaks of epidemic among all the older hares. Mr Frette believed it to be the lung disease described by Collett. In 1928 hares were scarce. By 1929 they had begun to increase again, and in 1930 they were numerous once more.

(b) Mr Claus Anderson of Tromsø informed me that outbreaks of epidemic disease take place among the hares on some of the islands off the coast, near Tromsø. These outbreaks apparently occur when the hares become too abundant.

(c) Mr Mathisen informed me that on an island in Porsanger Fjord (north Finmark), people were killing a great many hares every day in the autumn of 1930.

### 4. EPIDEMICS AMONG MOLES (*TALPA EUROPAEA*) IN THE SOUTH OF ENGLAND.

During the early summer of 1927 evidence was obtained of widespread mortality among moles in the country for about five miles round Oxford. A striking epidemic was observed by Mr F. J. Prewett on his farm at Tubney, Berks., during June. Moles had been abundant here for some time, and they suddenly began to come to the surface and die, with the result that after about a week very few were left on the farm. I found a recently dead mole at Cothill, near the last area mentioned, at the same time. Three specimens were examined bacteriologically by Dr A. D. Gardner, who has kindly communicated the following notes to me:

1. Mole found in dying condition by Mr Prewett on his farm, 24. vi. 1927.

Examined shortly after death. The lungs showed white-grey spots visible to the naked eye. A smear of the lungs showed no bacteria, cultures from heart blood, lungs and spleen were negative, nor was any parasite seen in blood smears. No special symptoms were noted.

2. Found dead by Mr Prewett at the Tubney farm, 24. vi. 1927. The lungs were in the same condition as in No. 1. All cultures were negative, as also were blood smears.

3. A lung from a specimen found dead at Cothill was sectioned and submitted to Dr Gardner.

In the lungs of all three specimens Dr Gardner observed small darkly staining bodies about the size of red-blood corpuscles. The nature of these was not determined.

A number of other dead moles were reported independently by various other observers in districts round Oxford. A summary of these records is given below: in no case were signs of external injury seen, as might have been expected had the moles been killed by human beings or animals or birds of prey.

*Shotover*, 15. vi. 1927. Dead mole on Johnston's Piece, Shotover Hill (Mr E. M. Nicholson).

*Baldon*, end of May, 1927. Moles were extremely abundant at the end of May, and then all disappeared within about a week, all being gone before the middle of June. In October, 1927, they had only just begun to return again, and one dead one was found lying on a mole hill at the beginning of this month (Mr D. Skilbeck).

*Boar's Hill*, beginning of June, 1927. Dr Gardner found a dead mole.

*Eynsham*, 30. vi. 1927. Three dead moles seen between Oxford and Eynsham, in the fields (Mr G. Tickner).

*Bagley Wood*, May and June, 1927. Dead moles were seen by me in various parts of the wood, in early May, June 7th, between June 20th and July 11th, and on July 11th (six altogether). In September, 1927, I noted that moles were scarce in the wood, where they had been abundant in the previous spring, and on October 6th I found another dead one. These observations are supported by those of other people. Prof. J. W. Munro saw three dead moles in the wood in mid-June, while Mr Cross, the gamekeeper, reported several in another part of the wood during the end of June and beginning of July.

Farther afield, there was also some slight evidence of reduction in numbers and deaths. Thus, I found a dead mole, apparently uninjured, near Kirtlington on 4. vi. 1927. Mr O. W. Richards found two dead moles near Cholsey (near Wallingford) on 25. v. 1927. In the New Forest, Dr T. G. Longstaff informed me that moles were very numerous in his garden at Picket Hill, Ringwood, in the early spring of 1927. When he returned in July, after an absence of three months, there were practically none left. Finally, a remarkable mortality was witnessed a year later (July, 1928) by Mr T. Bird, on a farm near Colchester, when moles were observed to come out in numbers and die on the surface. We are justified, then, in concluding that in May and June,

1927, there was considerable mortality among moles round Oxford, and apparently also in places farther away. There was also mortality a year later at Colchester. The examinations made by Dr Gardner point to some epidemic disease being partly responsible. It should be noted, however, that the summer of 1927 was very dry, and that the unusual conditions may possibly have had something to do with the mortality, if only by predisposing the moles to attack by disease. I am informed by Mr Gee, who has a farm near Oxford, that during the drought of 1921 moles came out and died and that when he gave water to one of them it lapped eagerly. It is of course well known that moles make runs to water. He believed that the dry conditions of 1929 were in some areas having a similar effect as he had seen two dead ones (2. vii. 1929). There is no evidence that drought in 1927 was anything like as severe as 1921, and I suggest provisionally that lack of water (which indirectly causes lack of food, through the ground being dry) may be a determining factor in starting epidemics among moles.

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# A COMPARISON OF EXTENDED AND SHORT METHODS IN THE CALCULATION OF A LIFE TABLE FOR MALES IN LONDON.

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THE last life table for the Administrative County of London was prepared by Mr King from the census population of 1911 and the deaths in 1910, 1911, 1912, and published in Part I of the Supplement to the *75th Annual Report of the Registrar-General*, 1914. The national life tables (No. 9), prepared by Sir Alfred Watson after the census of 1921, included one for "Greater London," the boundaries of which extend considerably beyond the County Council area and enclose the range of jurisdiction of the Metropolitan Police, so that this table is not geographically comparable with Mr King's 1911 table. The object of the present study was a comparison between the extended method of Mr King and the shorter ones of Drs Brownlee and Snow, to see whether the methods gave comparable results as regards the expectation of life, the question with which the *public health worker* is mainly concerned. As an example I selected the County of London (census population for 1921, deaths in 1920, 1921, and 1922) as affording, at the same time, an opportunity of making a table comparable with that which Mr King prepared for 1911. Drs Brownlee and Snow have already shown that their methods gave very satisfactory results as judged by the older life tables of this country. This paper will show that similar results have, in the main, been obtained as regards the 1921 experience.

The English life tables No. 8 (1911), prepared by Mr King, and No. 9 (1921), prepared by Sir Alfred Watson, were both based upon the population at the respective censuses and the deaths in the three years, centred on the year in which the census was taken. It was considered that this method gave a truer indication of the prevailing mortality experience of the community, in which the duration of life has been increasing continuously for several decades, than the earlier method of basing the table "upon two censuses and the deaths of the intervening ten years," which was followed in the preparation of No. 7 and the earlier life tables. For the same reason, as well as for the purpose of rendering the results comparable, it was desirable that the methods employed in the construction of the present life table should approximate, as far as possible, to those on which the 1911 and 1921 English tables were prepared.

For the extended table which I have constructed, I adopted Mr King's methods, a full explanation of which was given in the *Report of the Registrar-*

*General* mentioned above. In view of the remarks of Sir Alfred Watson regarding the fluctuations of the birth-rate during, and immediately succeeding, the period of the war, it was thought necessary to adopt his method for working out the  $q_x$ \* at the individual ages between 0 and 4 years. As the quarterly numbers of births for the separate sexes were not available for the County Council area, it was assumed that the sex ratio as given by the annual figures held good during the quarters. On this basis an estimate was made of the number of male births in each quarter. Subject to this limitation, the  $q_x$  has been worked out in accordance with the formulae given by Sir Alfred Watson for the ages 0, 1, 2, 3 and 4. He used the same method for age 5 also, but as separate figures for deaths at this age were not available for London, I have had to depend on the methods of graduation, to be mentioned later, for the  $q_x$  at this age. At the same time, another method was tested. Births from 1914 onwards were taken, and, by deducting the number of deaths among infants at the respective ages, populations aged 0, 1, 2, 3 and 4 at the beginning of 1920, 1921 and 1922 were arrived at. Thus, the arithmetical mean of the number of births in 1914 and 1915 was taken to represent the population of infants, aged 0, on January 1st, 1915 and, by subtracting from it the number of deaths of children, aged 0-1 in 1915, 1-2 in 1916, 2-3 in 1917, 3-4 in 1918, and 4-5 in 1919, the population entering on age 5 on January 1st, 1920 was calculated. By a similar process the populations entering on ages 0, 1, 2, 3 and 4 at the beginning of each of the three years, 1920, 1921 and 1922 were calculated. The total number of deaths of children at ages 0-1, 1-2, 2-3, 3-4 and 4-5 during the same years (1920, 1921, 1922), when expressed as a ratio of the populations calculated, gave the  $q_x$  for each of the respective ages. This method ignores immigration and emigration, but at these ages the error is probably too small to have any appreciable effect upon the results.

Two life tables were worked out, one utilising the  $q_x$  as found by the method just described and the other by Sir Alfred Watson's formulae. Table I shows the difference in expectation of life produced by the two methods. Sir Alfred Watson's method gives a very slightly longer mean length of life in each case. At birth it amounts to 0.12 of a year, nearly one month and thirteen days, but at the subsequent ages it is very much less. By the ninth year the difference has disappeared. From the fifth year onwards the deaths and the populations were grouped into quinquennial age periods up to age 100, and then in a single group of 100 and over. Utilising the methods described by Mr King these figures were graduated so as to secure central pivotal values of populations and deaths at the ages, 12, 17, etc., to 97. These population figures, on the addition of half the graduated deaths, gave  $l_x$ , the population at exact ages  $x$ , and the ratio of the graduated deaths to  $l_x$  gave the  $q_x$  at the respective quinquennial pivotal points, 12 to 97. By osculatory interpolation the value of  $q_x$  was obtained for the ages between 17 and 92. To complete the

\* This and the similar terms are explained at the end of the paper.

values for the period 5 to 16 the known values of  $q_x$  at 3, 4, 12, 17 and 18 were taken and Lagrange's formula was used for calculating  $q_x$ 's at ages 5, 6 and 7. Next, taking values at ages 4, 5, 6, 7 and 12 as  $u_1, u_2, u_3, u_4$  and  $u_5$ , the intervening values of  $u_x$  were worked out on the assumption that the fifth and subsequent differences were negligible. Similarly, with the known values of  $q_x$  at ages 9, 10, 11, 12 and 17 the  $q_x$ 's at ages 13, 14, 15 and 16 were calculated, the same process also being utilised for supplying these values at ages beyond 92. At 104 it was found that the value of  $q_x$  became negative and hence the life table has stopped with age 103.

Table I. *Comparison of expectation of life as obtained by Watson's method and an alternative method. Males. Administrative County of London.*

Age	Watson's Method (a)	Alternative method (described in the text) (b)	(a)-(b)
0	54.28	54.16	Nearly 1 month and 13 days
1	58.35	58.33	" 7 days
2	58.98	58.96	" 7 "
3	58.77	58.76	" 3.5 "
4	58.25	58.22	" 11 "
5	57.59	57.56	" 11 "
6	56.83	56.80	" 11 "
7	56.00	55.98	" 7 "
8	55.12	55.11	" 3.5 "
9	54.22	54.22	—
10	53.31	53.31	—

Mr King's extended method gives a smooth graduation and the process of construction has been so fully explained by him that there is little or no difficulty in understanding and applying it. It must be admitted, however, that it is a laborious process and, in this respect, the shorter methods devised by Drs Brownlee and Snow have their advantage. It is proposed, first, to indicate briefly the basis of these two methods and then to illustrate by tabular presentation the close relationship between the results deduced from them and those obtained by the extended method.

There are some points in common between these two short methods. The authors utilised the data given by the life tables, prepared by the extended method, as their basis and constructed empirical formulae which yield the expectation of life at the definite age periods with only a small margin of error from the figures in the extended tables. Dr Snow devised two functions,  ${}_np_x$  and  ${}_nk_x$ . The former expresses the probability of living for a period of  $n$  years after attaining age  $x$ , namely,  $\frac{l_{x+n}}{l_x}$  in terms of the death-rate for the age group. He evolved functional relationships, by the method of least squares, between the death-rate and  ${}_np_x$ , which, in some parts of the life table, were linear and in others parabolic of the second order. Towards the end of the life table, as the population rapidly decreased to extinction, the relationship was best expressed by a hyperbolic function of the form

$$p = ae^{-br},$$

so that the curve was made to asymptote to the  $x$ -axis.  ${}_nk_x$  is defined by the relation

$${}_nk_x = \frac{l_x + l_{x+1} + \dots + l_{x+n-1}}{l_x}.$$

Dr Snow established, again by the method of least squares, a relationship between  ${}_np_x$  and  ${}_nk_x$ . Thus, from known values of the former, the latter could be deduced. Tables have been prepared, which cover the entire field of death-rates likely to be met with, and have been published along with Dr Snow's lucid description of the whole process in Part II of the supplement to the *75th Annual Report of the Registrar-General, 1914*. The construction of life tables by this method has, therefore, been made sufficiently easy to be safe in the hands of public health workers.

Dr Brownlee first established that there existed a linear relationship between life table death-rate and standardised death-rate at the respective age periods. He worked at first with twelve tables which, "being constructed on different plans, probably represented varying degrees of accuracy." Mr Finch of the General Register Office subsequently repeated this work, utilising data from more recent life tables and evolved equations of relationship, which gave closer approximation. In his later work Dr Brownlee made use of this material and, at the same time, discovered that the use of a standard population, dying out in arithmetical progression, gave results which were eminently satisfactory.

For a full discussion of this method reference may be made to Dr Brownlee's paper in the *Journal of Hygiene*, xiii, 178 (July, 1913) on "Studies in the meaning and relationships of birth- and death-rates," and to his report to the Medical Research Council on "The use of death-rates as a measure of hygienic conditions" (*Special Report Series*, No. 60).

By applying either of these short methods the calculation of the expectation of life at different age periods will not take more than a couple of hours. However, it must be pointed out that there is a difference between the two short methods in that Dr Snow's method gives not only the expectation of life but also the number of survivors at quinquennial and decennial periods. Dr Brownlee's formulae apply solely to one particular section of a life table, *e.g.* the evaluation of the life table death-rate; knowing this function we can obtain the expectation of life from the relationship

$$E_x = \frac{1000}{\text{Life table death-rate}}.$$

For the sake of comparability the  $q_0$  of Dr Snow's method has been taken to be the same as that calculated for the extended table by Sir Alfred Watson's formula. In Dr Brownlee's method it is the death-rate for the age group 0-4 that is used. The figure has been obtained from the same formulae. It may be seen that, on the whole, the expectation of life is given with a sufficient degree of accuracy for most public health purposes, except perhaps at birth

and beyond 65. It has been stated by Dr Snow that, for early ages, even the extended method gave only results of doubtful accuracy.

To estimate the accuracy of the short methods when applied to the mortality of the whole country Brownlee's and Snow's equations were applied to the requisite mortality data for males in England and Wales during 1920-22. The results as will be observed in Table III are in close accord with those obtained by Sir Alfred Watson in his extended life table for the same period.

Table II. *Comparison of expectation of life obtained by Watson's extended method and the short methods of Brownlee and Snow. Males. Administrative County of London, 1921.*

Age	Extended method	Dr Snow's method	Dr Brownlee's method	Snow's deviation from extended method	Brownlee's deviation from extended method
0	54.28	53.47	53.08	- 0.81	- 1.20
5	57.59	57.25	57.25	- 0.34	- 0.34
10	53.31	53.25	53.33	- 0.06	+ 0.02
15	48.81	48.76	48.82	- 0.05	+ 0.01
20	44.52	44.46	44.46	- 0.06	- 0.06
25	40.30	40.24	40.25	- 0.06	- 0.05
35	31.91	31.83	31.89	- 0.08	- 0.02
45	24.08	24.01	24.07	- 0.07	- 0.01
55	16.93	16.98	17.05	+ 0.05	+ 0.12
65	10.94	10.98	11.07	+ 0.04	+ 0.13
75	6.38	6.31	6.52	- 0.07	+ 0.14
85	3.60	3.33	—	- 0.27	—
and upwards					

Table III. *Comparison of expectation of life obtained by Watson's extended method and the short methods of Brownlee and Snow. Males. England and Wales, 1921.*

Age	Extended method	Dr Snow's method	Dr Brownlee's method	Snow's deviation from extended method	Brownlee's deviation from extended method
0	55.62	56.07	55.04	+ 0.45	- 0.58
5	58.81	59.28	58.89	+ 0.47	+ 0.08
10	54.64	55.17	54.95	+ 0.53	+ 0.31
15	50.12	50.21	50.33	+ 0.09	+ 0.21
20	45.78	45.89	45.96	+ 0.11	+ 0.18
25	41.60	41.63	41.72	+ 0.03	+ 0.12
35	33.25	33.28	33.38	+ 0.03	+ 0.13
45	25.22	25.26	25.34	+ 0.04	+ 0.12
55	17.73	17.77	17.86	+ 0.04	+ 0.13
65	11.36	11.39	11.50	+ 0.03	+ 0.14
75	6.59	6.55	6.77	- 0.04	+ 0.18
85	3.72	3.55	—	- 0.17	—
and upwards					

In both tables Dr Snow's method gives a better fit for some age periods and Dr Brownlee's for others.

It is interesting to compare the increase in expectation of life that the County of London attained between the years 1911 and 1921. The 1911 figures have been taken from Mr King's table. It starts with age 12 and a comparison of the earlier years is, therefore, impossible. It may be seen that there has



been a definite increase at every age except 85, when there is a slight decrease. It has been said that the accuracy of the mortality figures at the later age periods is questionable and this reason may explain the decrease.

Table IV. *Comparison of the expectation of life of Males, Administrative County of London, in 1911 and 1921.*

Age	1911	1921	Increase of expectation 1921 - 1911
12	49.43	51.49	2 years
15	46.74	48.81	2 years
20	43.35	44.52	1½ years
25	38.06	40.30	2¼ years
35	29.84	31.91	2 years
45	22.45	24.08	1½ years
55	15.95	16.93	1 year
65	10.51	10.94	5 months
75	6.28	6.38	1 month
85	3.71	3.60	Decrease of 1 month
95	2.19	2.38	Increase of 2 months

Lastly, I must acknowledge the help and guidance which Mr W. T. Russell, of the Epidemiological Section of the London School of Hygiene and Tropical Medicine, has so generously given me throughout the preparation of this life table.

In Table IV the values of the expectation of life as obtained by the extended method have been given only for specific ages. In case the values are required for individual ages Table V gives a detailed presentation of the facts.

Table V. *Life table. London Administrative Area. Males, 1920-22.*

Age	$l_x$	$d_x$	Complete expectation of life	Age	$l_x$	$d_x$	Complete expectation of life
0	1,000,000	86,122	54.28	29	804,006	3,398	36.91
1	913,878	25,008	58.35	30	801,208	3,541	36.06
2	888,870	11,842	58.98	31	797,667	3,711	35.22
3	877,028	7,157	58.77	32	793,956	3,909	34.38
4	869,871	5,081	58.25	33	790,047	4,151	33.55
5	864,790	3,603	57.59	34	785,896	4,432	32.73
6	861,187	2,589	56.83	35	781,464	4,731	31.91
7	858,598	1,937	56.00	36	776,733	5,029	31.10
8	856,661	1,563	55.12	37	771,704	5,305	30.30
9	855,098	1,400	54.22	38	766,399	5,546	29.50
10	853,698	1,390	53.31	39	760,853	5,763	28.71
11	852,308	1,485	52.40	40	755,090	5,980	27.93
12	850,823	1,645	51.49	41	749,110	6,401	27.15
13	849,178	1,838	50.58	42	742,709	6,485	26.38
14	847,340	2,040	49.69	43	736,224	6,784	25.61
15	845,300	2,233	48.81	44	729,440	7,096	24.84
16	843,067	2,407	47.94	45	722,344	7,434	24.08
17	840,660	2,560	47.08	46	714,910	7,809	23.32
18	838,100	2,697	46.22	47	707,101	8,232	22.58
19	835,403	2,819	45.37	48	698,869	8,699	21.84
20	832,584	2,924	44.52	49	690,170	9,197	21.10
21	829,660	3,013	43.67	50	680,973	9,730	20.38
22	826,647	3,084	42.83	51	671,243	10,298	19.67
23	823,563	3,120	41.99	52	660,945	10,902	18.97
24	820,443	3,123	41.15	53	650,043	11,536	18.23
25	817,320	3,118	40.30	54	638,507	12,192	17.60
26	814,202	3,131	39.45	55	626,315	12,871	16.93
27	811,071	3,185	38.60	56	613,444	13,573	16.28
28	807,886	3,280	37.75	57	599,871	14,296	15.64

Complete expectation of life			Complete expectation of life		
Age	$l_x$	$d_x$	Age	$l_x$	$d_x$
58	585,575	15,029	82	92,488	15,524
59	570,546	15,761	83	76,964	13,842
60	554,785	16,494	84	63,122	12,156
61	538,291	17,227	85	50,966	10,495
62	521,064	17,959	86	40,471	8,894
63	503,105	18,641	87	31,577	7,387
64	484,464	19,259	88	24,190	6,029
65	465,205	19,864	89	18,161	4,828
66	445,341	20,496	90	13,333	3,768
67	424,845	21,191	91	9,565	2,852
68	403,654	21,954	92	6,713	2,086
69	381,700	22,716	93	4,627	1,480
70	358,984	23,395	94	3,147	1,023
71	335,589	23,914	95	2,124	696
72	311,675	24,207	96	1,428	470
73	287,468	24,276	97	958	319
74	263,192	24,141	98	639	219
75	239,051	23,782	99	420	153
76	215,269	23,188	100	267	108
77	192,081	22,356	101	159	76
78	169,725	21,311	102	83	51
79	148,414	20,082	103	32	27
80	128,332	18,688	104	5	—
81	109,644	17,156			

## KEY TO THE NOTATION.

$q_x$ : The probability of dying in a year. The ratio of the total number of deaths in the year,  $x$ , to the population entering on that year.

$l_x$ : The population attaining age  $x$  at the beginning of the year.

${}_np_x$ : The probability of living  $n$  years for the population entering on age  $x$ .

${}_nk_x$ :  $\frac{l_x + l_{x+1} + \dots + l_{x+n-1}}{l_x}$ .

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# ADSORPTION OF TUBERCULIN BY COAL DUST.

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WITH AN ANALYTICAL NOTE BY MR E. T. WATERS, PH.D., B.Sc.

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WHILE investigating the effects of silica dust and coal dust respectively in the production of respiratory disease in coal miners, we have been struck by the sharp contrast between silicotic coal miners in South Wales as compared with silicotic gold miners in South Africa in their respective liability to pulmonary tuberculosis. Coal miners have always been noted for their relatively low tuberculosis mortality, while gold miners and other workers in hard rock, but exempt from simultaneous exposure to coal dust, are conspicuous for their marked liability to fatal pulmonary tuberculosis in late middle age. And yet the recent findings of Cummins and Sladden (1930) and of the Medical Staff of the Welsh National Memorial Association (1930) indicate that both in the pathological, histological and chemical characters of the lungs of Welsh coal miners and in the X-ray appearances observed in long-service colliers of over forty years of age, there is nothing to distinguish them from Rand gold miners suffering from silicosis except the added presence of large amounts of coal dust in the lung tissue of coal miners and their relatively low tuberculosis mortality. This contrast has been discussed by one of us (S.L.C.) in a recent paper (1931) and the suggestion made that the well-known adsorption power of finely divided carbon particles for colloidal substances might be a factor in reducing the liability of the coal miner to pulmonary tuberculosis.

The quantity of "coaly material" accumulating in the lungs of anthracite coal miners is sometimes very large. In the two most marked cases reported by Cummins and Sladden (*loc. cit.*) the coaly material amounted to 119 grm. and 104 grm. respectively, and the average for twenty-two coal miners was 33 grm., whereas in the lungs of five male adults living under industrial conditions but unconnected with coal-mining or trimming, the coaly material in the lungs varied from 0.18 to 1 grm. These figures will serve to show that in coal miners, as opposed to ordinary town dwellers, there is likely to be sufficient coal dust, in a state of very fine division, to determine adsorption effects.

As to the adsorption of the active principle of tubercle bacilli by carbon, while there have been differences of opinion amongst those studying the question, recent observations have established the activity of charcoal in this respect. Long and Seibert (1925) were unable to demonstrate any adsorption

of the active principle of tuberculin by animal charcoal. Later, however, Boquet, Nègre and Valtis (1928) showed conclusively that a finely divided charcoal, *norit supra*, is very potent in adsorbing from tuberculin the constituent on which its activity depends.

Dorset, Henley and Moskey (1927), too, proved by careful experiments that various adsorbents carrying a negative charge, including charcoal, could largely or completely abolish the activity of tuberculin.

These experiments had been carried out on guinea-pigs and in none of them was coal dust employed, so we decided to investigate the adsorptive power of coal dust and to test the activity of the tuberculin before and after contact with the dust, by the intradermal method in tuberculous patients.

*Exp. 1.* A small lump of anthracite coal, as used in heating the Laboratory, was ground to a fine powder in a mortar and suspended in normal saline solution, the suspension being sterilised in the autoclave.

Two dilutions of "Old Tuberculin" (No. 1586 B.W. & Co.) were prepared in saline, one of a strength of 1 in 500, the other 1 in 2500. A sample of each dilution was mixed with an equal volume of the suspension of coal dust and the mixtures were left in contact overnight at room temperature. Each tuberculin dilution was, similarly, mixed with an equal volume of sterile normal saline solution and left overnight at room temperature for use as a non-adsorbed "control."

Next day, the carbon-tuberculin mixtures were shaken, centrifuged, and the clear supernatant fluid pipetted off for use. Thus we had available for use two samples of 1 in 1000 tuberculin and two of 1 in 5000 tuberculin, one of each being "adsorbed" and the others "non-adsorbed."

These samples we tested on adult male tuberculous patients, by the intradermal method, sufficient being introduced into the skin to produce a "wheal" about the size of a threepenny piece. The results, read after 48 hours, are recorded in Table I.

Table I. *Exp. 1.*

Patient's initials	Tuberculin 1 in 1000		Tuberculin 1 in 5000	
	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.
W.	11	20	—	—
J.	9	20	—	—
Wa.	12	16	—	—
L.	11	15	—	—
M.	7	20	—	—
D.	—	—	0	18
McA.	—	—	0	17
Jn.	—	—	0	18
J.E.	—	—	0	25
R.	—	—	10	30

It will be seen that the amount of active substance in the 1 in 1000 dilution had in all cases been diminished, while it had been completely abolished in four out of the five patients tested with the 1 in 5000 dilution of tuberculin.

*Exp. 2.* It was decided to repeat the above experiment, approximating more closely to body fluid conditions by making the contact between the coal dust and the tuberculin in a "buffered" solution with a slightly alkaline reaction. This was accomplished by diluting the tuberculin in a solution of 4 per cent. disodium phosphate, the coal dust being suspended in saline as before. The final solutions, both the adsorbed and the non-adsorbed, were as before in tuberculin concentration, but contained 2 per cent. of disodium phosphate.

The adsorbed and non-adsorbed tuberculin solutions were tested on twenty patients as before. The results are recorded in Table II.

Table II. *Exp. 2.*

Patient's initials	Tuberculin 1 in 1000		Tuberculin 1 in 5000	
	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.
Cr.	0	18	—	—
R.T.	0	16	—	—
W.	0	23	—	—
Ab.	0	23	—	—
B.	0	17	—	—
G.	0	12	—	—
K.	0	12	—	—
H.	0	12	—	—
H	0	12	—	—
Martin	0	Slight	—	—
A.L.	0	23	—	—
S.R.	—	—	0	0
J.P.	—	—	0	12
W.T.J.	—	—	0	12
H.	—	—	0	20
M.	—	—	0	13
Bu.	—	—	0	16
Wi.	—	—	0	15
Dk.	—	—	0	0
Ja.	—	—	0	15

In this experiment, thanks doubtless to the slightly alkaline reaction, the adsorption sufficed to annul all activity in the tuberculin dilutions exposed to coal dust.

Taken by themselves, Exps. 1 and 2 suggested strongly that anthracite coal dust might be a potent factor in inhibiting or diminishing the activity of the products of growth and disintegration of tubercle bacilli in the tissues. There remained, however, the question whether the massive collections of coal dust in the lungs of miners, situated as they are close to the apices, under the pleura, in the tracheo-bronchial glands and along the peribronchial and periarterial lymphatics, in the areas characteristically liable to tuberculous lesions, might not still be rendered inactive as adsorbents by previous saturation with the colloids of the body fluids.

This consideration raised the question of the extent to which previous saturation with one substance is able to annul the adsorption power of coal dust for another. We decided to make a preliminary test as follows:

*Exp. 3.* A thick suspension of anthracite coal dust in saline was prepared and to 3 c.c. of this was added 3 c.c. of fresh human blood serum. The mixture was left at 37° C. for 3½ hours, being shaken up at intervals. The coal dust was then centrifuged down, washed three times in saline, and suspended in the phosphate solution. Tuberculin dilutions of 1 in 1000 and 1 in 5000 were prepared as before by mixing with equal volumes of the serum-saturated coal-dust residue resuspended in phosphate solution; while "control" tuberculin dilutions were made up with phosphate solution alone.

These preparations were left overnight at room temperature and compared by intradermal testing next day after the bulk of the coal dust had been removed by centrifugation. Here, however, a slight difficulty arose, as it proved impossible, with the electric centrifuge used, to get rid of all the coal dust. Contact with serum had apparently so altered things that the dust particles, instead of tending to spontaneous deposit, made a very homogeneous and stable suspension and resisted even fairly prolonged centrifugation.

These intradermal tests proved inconclusive; partly perhaps owing to the imperfect "clearing" of the solutions and partly because the syringe prepared for inoculating the 1 in 5000 "non-adsorbed" dilutions proved defective so that it was not possible to get the quantities correct owing to leakage.

The results of these tests, carried out on eighteen patients, are recorded in Table III.

Table III. *Exp. 3.*

Patient's initials	Tuberculin 1 in 1000		Tuberculin 1 in 5000	
	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.
H.J.L.	10	15	—	—
W.M.	0	12	—	—
G.H.	14	14	—	—
H.R.	15	8	—	—
B.J.	8	10	—	—
T.Y.	20	20	—	—
G.C.	6	9	—	—
L.Y.	16	22	—	—
Dn.	12	20	—	—
K.	0	0	—	—
Hy.	12	12	—	—
T.R.	10	14	—	—
M.D.	—	—	0	0
S.P.	—	—	24	20
E.G.H.	—	—	Slight	Slight
L.M.	—	—	12	16
S.L.	—	—	20	12
R.B.	—	—	8	13

Although the results were, on the whole, suggestive of some further adsorption of tuberculin in a majority of the tests, yet there were three anomalous cases in which the "adsorbed" gave a larger reaction than the "non-adsorbed" tuberculin. It is true that two of these were in the 1 in 5000 group, in which the correct quantities of the non-adsorbed tuberculin were not introduced as

explained above; but there remained the possibility of some non-specific irritation effect due to imperfect centrifugation and perhaps the retention of some of the serum used in the preliminary adsorption.

The results of Exp. 3 were regarded as inconclusive and as requiring repetition.

*Exp. 4.* The details of this experiment were identical with those of Exp. 3, except that the serum and coal dust were left in contact for 15 minutes only, the period found sufficient for adsorption by Dorset, Henley and Moskey (1927), instead of for 3½ hours as in Exp. 3. Further, the final products, after tuberculin adsorption, were spun down in a much more powerful centrifuge and were almost completely cleared of suspended carbon, though the same tendency of the carbon to remain in suspension after treatment with serum was noted as in Exp. 3.

The results, in the fourteen female patients tested, are recorded in Table IV.

Table IV. *Exp. 4.*

Patient's initials	Tuberculin 1 in 1000		Tuberculin 1 in 5000	
	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.
R.P.	20	35	—	—
P.M.P.	15	25	—	—
L.C.	0	20	—	—
M.W.	15	25	—	—
L.M.F.	15	20	—	—
M.F.	20	35	—	—
B.J.	15	30	—	—
M.E.	17	20	—	—
E.P.	—	—	5	12
M.H.	—	—	0	12
K.H.	—	—	0	0
B.C.	—	—	12	20
M.E.	—	—	0	20
D.L.	—	—	0	25

Here the results were invariably indicative of a stronger reaction to the "non-adsorbed" tuberculin. It was quite clear that previous contact with serum had not completely saturated the carbon in respect of its power of adsorbing tuberculin.

In this experiment, the possibility of non-specific effects was excluded by inoculating the supernatant fluid, obtained after washing the carbon free from serum, intradermally into six tuberculous patients, with negative results. It occurred to us, however, that the sharper results of Exp. 4 as compared with Exp. 3 might be due to a less complete adsorption of serum proteids owing to the shorter period, 15 minutes only, allowed for contact between the serum and the coal dust. This question was only capable of solution by a repetition of the test and this is described under Exp. 5. It was thought worth while, however, to estimate the total nitrogen in a sample of the serum-adsorbed coal dust left over from the experiment and to compare it with the

total nitrogen in the suspension of coal dust in saline previous to contact with serum. This test, suggested by Mr E. T. Waters, Ph.D., B.Sc., a Biochemist working in the Central Tuberculosis Laboratory with a grant from the Medical Research Council, was executed by him, and the results show, at least, that a significant though small amount of nitrogenous substance had been adsorbed prior to the exposure of the dust to tuberculin. Mr Waters gives his results as follows:

"In order to establish the fact that the coal dust actually adsorbed some of the constituents of the serum under the experimental conditions, and also to obtain an approximate figure of the amounts so adsorbed, if any, it was decided to determine the nitrogen contents of the original coal and of the resulting coal-adsorbate complexes, in the two series of experiments, viz. Exps. 4 and 5.

The various samples were washed free from saline, spun down on the centrifuge, and dried *in vacuo* over phosphorus pentoxide. The nitrogen estimations were carried out by the Kjeldahl method, on amounts of material ranging from 30 to 100 mgm. Several investigators have reported anomalous results in the total nitrogen estimation of coal by the Kjeldahl method. Erratic low results were also obtained by the writer until the preliminary sulphuric acid digestions, carried out as described below, were continued over a prolonged period. Hence the following details are recorded:

The digestions were conducted in small pyrex flasks, employing 2 to 3 c.c. of concentrated sulphuric acid and a small amount of copper sulphate. The mixtures were heated, gently for a few hours then more strongly until they boiled vigorously. This treatment was continued long after the solutions became clear; failure to do so resulted in low nitrogen figures. Thus the digestions were continued for at least 24 hours, and occasionally for 2 days. Consistent nitrogen percentages were then obtained.

### Results.

#### Series 1.

Coal dust only, N = 1.46 %	
Coal-adsorbate complex after serum and "1 in 1000" tuberculin, N = 1.56 %	
" " "1 in 5000" " N = 1.55 %	

#### Series 2.

Coal only, N = 1.46 %	
Coal-adsorbate complex after serum only, N = 1.54 %	
" " and "1 in 1000" tuberculin, N = 1.54 %	
" " "1 in 5000" " N = 1.54 %	

It is to be noted that the nitrogen adsorbed from these tuberculin solutions would hardly reach a detectable amount.

Thus a nitrogen 'difference' of 0.08 per cent. is obtained. Assuming the material adsorbed to be of protein nature and multiplying by the customary factor 6.25, the amount of adsorbed material is approximately 0.5 per cent. of the weight of coal dust used. Treatment with serum in Series 1 took place over a period of 15 minutes at 37° C., that of Series 2 over a period of 24 hours



at room temperature. The above analytical figures indicate no increase in adsorption with extended contact with serum, hence it would appear that in the above experiments the free surface of the coal was saturated with respect to serum proteins."

There remained, however, the possibility that a longer period of exposure of the coal dust to the serum might be found, in biological tests, to lead to a more marked saturation of the dust with protein and to a loss of power to adsorb tuberculin. We accordingly repeated Exp. 4 with the necessary modification to throw light on this point.

*Exp. 5.* Details were as in Exp. 4, but the serum was left in contact with the coal dust overnight at room temperature, and higher dilutions of tuberculin were used. The results are recorded in Table V.

Table V. *Exp. 5.*

Patient's initials	Tuberculin 1 in 5000		Tuberculin 1 in 10,000	
	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.	Adsorbed Reaction size in mm.	Non-adsorbed Reaction size in mm.
Ga.	10	16	—	—
W.Tr.	14	14	—	—
J.D.	8	14	—	—
G.W.	0	20	—	—
F.C.	0	0	—	—
H.H.	—	—	0	0
W.T.J.	—	—	12	14
T.	—	—	8	14
I.Y.	—	—	5	12
A.	—	—	10	20
T.E.	—	—	0	9

In this test, out of nine patients found capable of reacting to the tuberculin dilutions used, there was evidence of some degree of "adsorption" of the active principle of tuberculin in eight.

The possibility of non-specific effects was again excluded by testing the "washings" from the serum-coal dust mixture intradermally in six patients with completely negative results.

The weight of coal dust per volume of suspension was, in Exps. 4 and 5, 0.5 gm. in 1 c.c. The amount used in the previous experiments was approximately the same.

#### DISCUSSION.

While our experiments appear to establish the fact that anthracite coal dust is able to adsorb a considerable amount of the active principle of tuberculin, it is to be noted that, limited by what was justifiable in tests on active cases, we worked with very dilute solutions of tuberculin only. Dorset, Henley and Moskey, who used a charcoal, "norite," in their investigations, were able to remove all activity from so strong a concentration of tuberculin as 1 in 20; but it may be assumed that charcoal is a more powerful adsorbent than coal dust.

The chemical analysis described by Mr Waters shows that only a very small amount of serum proteid was adsorbed on to the coal dust, suggesting that the latter is not a very powerful adsorbent. Had charcoal been used, the amount would probably have been greater.

It will be noted that the most complete adsorption of the active principle of tuberculin was attained in Exp. 2, where the contact of fresh coal dust with tuberculin was brought about in a slightly alkaline medium.

The results after previous contact of the coal dust with serum, although proving that the dust still retained a considerable measure of its power to adsorb the active principle of tuberculin, were less sharp and indicated that the exposure to serum had reduced, though not abolished, the adsorption power.

While it is clear that tests of a different kind, involving the production of silico-anthracosis in laboratory animals, are necessary to settle the question whether the power of finely divided coal dust to adsorb the active principle of tubercle bacillary protein is capable of playing a part in the relative exemption of South Wales coal miners from the high incidence of late middle age tuberculosis common in other occupations involving exposure to the dust of hard stone, we think the results of the experiments here described sufficiently suggestive to justify further investigation in this direction.

#### CONCLUSIONS.

1. Anthracite coal dust is capable of adsorbing the active principle of tuberculin.

2. This power of adsorption is especially well marked when the contact between the coal dust and the tuberculin is brought about in a slightly alkaline medium.

3. Previous saturation with serum proteid, while it appears to diminish, does not annul the power of coal dust to adsorb the active principle of tuberculin.

Our thanks are due to Drs J. Brownlee and W. Davies, the Medical Superintendents of Glan Ely Hospital and Cefn Mably Hospital, where the tests were carried out, and to the Medical Research Council for a grant facilitating the investigation here recorded.

#### REFERENCES.

- BOQUET, A., NÈGRE, L. and VALTIS, J. (1928). *Compt. Rend. Soc. Biol.* **99**, 9.  
CUMMINS, S. L. (31. I. 1931). *Lancet*, i, 235.  
CUMMINS, S. L. and SLADDEN, A. F. (1930). *J. Path. and Bact.* **33**, 1095.  
DORSET, H., HENLEY, R. R. and MOSKEY, H. E. (1927). *J. Amer. Vet. Med. Assoc.* **70**, 373.  
LONG, E. R. and SEIBERT, F. B. (1925). *J. Amer. Med. Assoc.* **85**, 650.  
WELSH NATIONAL MEMORIAL ASSOC. (1930). *XVIII Annual Report for year ending March 31st, 1930*, p. 158.

# THE TECHNIQUE OF DETECTING DIPHTHERIA BACILLUS CARRIERS AND ITS APPLICATION.

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## INTRODUCTION.

THE modern methods of preventing diphtheria include the detection and isolation of the virulent diphtheria bacillus carrier, the Schick testing of individuals to distinguish susceptibles from non-susceptibles, the active immunisation of susceptibles with toxin antitoxin mixture or one of its modifications, and occasionally, when circumstances seem to demand it, the administration of antitoxin to confer passive immunity sufficient to tide the individual over an epidemic. Theoretically it would be possible to stamp out diphtheria by adopting either wholesale isolation of carriers or active immunisation of all susceptibles. In practice, however, isolation of all carriers is impossible, except in limited communities such as schools, ships, asylums, jails and hospitals, where it may be attempted with a reasonable prospect of success. It is in fact in such communities that the method of isolating carriers is particularly applicable in order to check sporadic outbreaks of diphtheria, especially where the proportion of susceptible individuals to non-susceptibles is high, as for example in an infants' school. Its application to the control of epidemics and to lowering the endemic level of diphtheria incidence does not appear to have met with success, although some Public Health authorities pursue a policy of isolating carriers in addition to immunising susceptibles. It is obvious that the success or failure of the isolation method will depend very largely upon the quality of the technique employed in detecting the carriers. It is proposed in what follows to elaborate in some detail the technique which should be employed for this purpose and to consider its application in certain specific instances.

Despite the truly vast proportions of the literature on diphtheria and the many papers which have appeared from time to time dealing with the carrier rate, there does not appear to be a single full account of the methods to be employed in detecting carriers. There is no doubt that a trained bacteriologist would find little difficulty in evolving an ample and satisfactory technique for himself if called upon to do so, but for the many who have had no special training in this aspect of bacteriology there is no guide as to how the best results may be obtained. The report of the Bacteriological Committee of the Medical Research Council in 1923, which is regarded as being the standard work on the subject, does not refer to the technique, and I have been unable to find any account of it in the literature since then. Owing to this omission,

unless a person called upon to make an examination for carriers has had some previous experience in that work he may be at a complete loss as to the best technique to employ. It is proposed to deal first with the methods which should be followed, to illustrate by examples the results which may be obtained with them, and then to consider some of their applications.

#### PRELIMINARY OBSERVATIONS.

For practical purposes diphtheria carriers are of three kinds, as classified anatomically: nose, throat and ear carriers. With regard to the last, the aural carrier state is almost always, if not always, associated with pathological conditions of the ear accompanied by a discharge and it is consequently only in the presence of such a discharge that ear swabs need be taken. Both nose and throat swabs, however, must be taken in every case, as the frequency of the nasal carrier is just as great as that of the throat carrier. This may be readily appreciated from the following table which gives the anatomical distribution of the organisms in 50 carriers found by me (1931) among 1000 Dublin school children, swabs being taken from both nose and throat.

Site	Positive cultures
Nose only	25
Throat only	18
Nose and throat	7
Total	<u>50</u>

The diphtheria bacillus is therefore to be sought in the nose and throat as a routine, and, when conditions warrant it, in the ear as well.

There are two types of diphtheria bacillus which may be found in these situations, either the virulent or the non-virulent type. Carriers of the non-virulent organism are not a menace to the community and do not require isolation and treatment as carriers of the virulent type do. The two types of carriers have, in consequence, to be distinguished from one another. This is done by isolating the organisms in pure culture and testing their toxin-producing powers by animal inoculation. The complete detection of the carrier, therefore, involves not only the successful culture of the diphtheria bacillus removed from the nose, throat or ear by means of a swab, and its identification by microscopical appearances, but also its isolation in pure culture and its final study by fermentation and virulence tests. If the isolation of carriers is to be both practicable and efficient their detection should involve as far as possible only one swabbing and the technique employed should therefore aim at securing 100 per cent. isolations of the diphtheria bacilli in the swabs.

From these preliminary considerations we may turn next to the details of the technique to be employed in order to achieve the desired results.

#### TECHNIQUE OF SWABBING.

The requirements at the time of swabbing are a small table, a chair, an ample supply of swabs and spatulas, a sufficiency of slopes of Loeffler's serum

for making primary cultures, a jar of 5 per cent. lysol and pen and paper for taking names. The slopes of Loeffler's serum should all be supplied with blank labels and three different coloured wools should be used to plug them to distinguish nose, throat and ear cultures. For example, an equal number of slopes with blue and white plugs may be provided for nose and throat cultures and a smaller number with green plugs for ear cultures.

For mass swabbings the swabs may be wrapped in white paper, a pair of swabs being enclosed in each wrapper. The requisite number of swabs to cover all purposes is then made into a package which is sterilised in the autoclave. This is much the most convenient way of carrying the swabs, as they occupy little space and their sterility is ensured, each pair being opened as it is required. For each throat swab a sterile spatula must be provided. It is best to wrap the spatulas in paper and to autoclave them also, opening each as it is required when the swab is being taken. The person taking the swabs should sit or stand at the table with the windows, open if circumstances permit, upon his right-hand side. On the table in front of him are placed the swabs, spatulas, tubes of Loeffler's serum and sheets of paper for taking names. He is approached from the left by the person to be swabbed and, having ascertained the name, enters it opposite the number of the examination. It greatly facilitates matters, particularly in dealing with young children, to have a teacher or other suitable person to take the names. The number of the examination is entered upon the labels on the serum tubes. As the swabs are taken they are well rubbed over the surface of the serum slopes, care being taken to assign each swab to a tube with wool of the correct colour. The jar of lysol is convenient to receive the used swabs and spatulas.

The position of the operator as described is worthy of note. He is situated between the person to be swabbed and the open window which is conducive to his comfort and lessens the risk of diphtheria bacilli being sprayed on to the various articles upon the table. Furthermore the light is to the best advantage and his right hand is in the most convenient position for writing and other movements.

In taking throat swabs care should be exercised that the swab comes in contact with as large a surface of the mucous membrane of the fauces and pharynx as possible, since there is no guide as to the situation in which the diphtheria bacilli may be found such as is present in cases of the disease. Similarly in taking nasal swabs, the swab should be inserted well into each nostril in turn and gently though firmly rubbed over the surface of the nasal mucous membrane. The same principles apply to the taking of aural swabs when they are necessary.

It is a great advantage to inoculate the swabs on to Loeffler's medium as soon as they have been taken. It prevents loss by drying and by outgrowth of the diphtheria bacilli by saprophytic organisms. Both these causes of loss are very considerable in their effects if some time must elapse before the swabs can be taken to the laboratory. It furthermore increases convenience

without increasing the bulk of the material to be handled. It is more economical, decreases labour, saves time and makes negligible the risk of clerical errors. If the swabs were to be taken to the laboratory prior to inoculation it would involve the use of tubes just as large as those required for serum and would mean, in addition, the labelling of two sets of tubes, first the swabs and second the culture tubes, so increasing the risk of errors.

#### TECHNIQUE OF DETECTION, ISOLATION AND IDENTIFICATION OF THE DIPHTHERIA BACILLUS.

The use of Allison and Ayling's medium (1929) as a routine method of isolating the diphtheria bacillus in pure culture, from the primary growths on serum, is to be advocated. It may be well, therefore, to consider first the preparation of this medium and to discuss the results which it may be expected to give and to deal with its limitations and how they may be overcome. The conclusions with regard to its uses and limitations are based on 2000 inoculations made on the medium from primary serum growths of material taken from the nose and throat.

Allison and Ayling's medium is a modification of Douglas's trypsinised serum tellurite medium. For its preparation the following reagents are employed:

(i) A 2 per cent. agar of pH 8 with a basis of either Hartley's digest broth or 1 per cent. peptone broth containing meat extract or Lab. Lemco in the usual proportions.

(ii) Sterile trypsinised tellurite serum.

(iii) 10 per cent. copper sulphate solution in distilled water.

The agar may be conveniently sterilised and stored for use in milk bottles as supplied by United Glass Bottle Manufacturers, Ltd., 400 c.c. of agar being contained in each bottle. The trypsinised tellurite serum is prepared by adding for each 100 c.c. of horse serum 8 c.c. of Allen and Hanbury's Liquor Trypsinae Co. and 10 c.c. of a 2 per cent. aqueous solution of potassium tellurite. The serum is then filtered with aseptic precautions through a Seitz filter after which it may be transferred to sterile tubes and placed in the cold room where it will keep for several months. A large quantity may, therefore, be prepared at one time and stored until required. The tellurite causes a precipitate in the serum which appears to be due to the formation of the insoluble calcium tellurite. This produces a deposit in the tubes and they should consequently be warmed to 55° C. for a few minutes and well shaken up before use, to disperse the precipitate. The copper sulphate recommended by Allison (1930) is the B.D.H. copper sulphate A.R.

When the medium is required for use it may be made as follows. To every 100 c.c. of nutrient agar melted and cooled to 55° C., 10 c.c. of the sterile trypsinised tellurite serum and 0.5 c.c. of the 10 per cent. copper sulphate are added. After mixing thoroughly the medium is poured into sterile Petri dishes and, when set, is placed in the incubator to dry preparatory to use.

The chief claims which have been made for this medium may be summarised briefly as follows:

(i) It prevents the growth of staphylococci and streptococci and allows the diphtheria bacillus and members of the diphtheroid group to grow unrestricted.

(ii) Spreading organisms of the *B. proteus* type, which are frequently present in the ear, are restricted in the limits of their growth to the formation of colonies.

(iii) The colony forms of the diphtheria bacillus and the diphtheroids are more characteristic than if copper sulphate is omitted from the medium, and in consequence colonies of the various members of the group may be distinguished from one another, leading to ready isolation of the diphtheria bacillus. The latter organism is said to form three distinct types of colony after 24 hours' incubation.

(a) A smooth glistening colony having a diameter of 0.75 to 2 mm. with a deep black central zone and a light grey peripheral belt sharply differentiated from the central area. The colony may or may not show a cup-shaped depression in the centre.

(b) Round glistening colonies about 0.5 mm. in diameter which are grey in colour.

(c) Dry, lustreless, flat colonies with an irregular margin and rough surface. They vary in size from 0.25 to 2.0 mm. and have a black centre with a greyish white peripheral zone.

(iv) A characteristic odour is said to be produced by the growth of the diphtheria bacillus.

For a full description of the colony forms of the diphtheria bacillus, Hofmann's bacillus and the other diphtheroids in addition to the various characteristics of the medium, the original articles should be consulted.

To deal with the first and second of the above claims, there is no doubt that the medium effectively suppresses or limits the growth of most organisms except those of the group to which the diphtheria bacillus belongs. In addition, such organisms as will grow, produce in general either white, yellow or brown colonies which cannot be confused with the colonies of the corynebacteria. It has been possible to confirm the authors' observations as to the nature of the unwanted organisms capable of growing on the medium, but it has been found in addition that there is a type of streptococcus which produces a flat white lustrous colony about 0.5 mm. in diameter which they do not mention and a number of moulds of various sorts. Although it is true to say that the majority of diphtheria bacilli are not inhibited in their growth by the presence of copper sulphate in the medium, none the less some are either inhibited for as long as 48 hours or else are completely suppressed. This suppression takes place even though the copper sulphate is present in only just sufficient quantity to achieve its object, namely, the inhibition of the organisms which by their luxuriant growth render the isolation of the diphtheria bacillus

difficult or impossible. Allison (1930), in his later communication, shows that different brands of copper sulphate vary so greatly in their inhibiting power that some are five times as potent as others. This would seem to suggest that it is not the copper sulphate which is active but rather some impurity associated with it. The suppression of certain strains of diphtheria bacilli by the medium does not constitute a very serious drawback to its use, as this may be allowed for in cases where the diphtheria bacillus is known to be present by adopting another method of isolating the organism in conjunction with the special medium.

With regard to the third claim outlined above, the first type of colony described is undoubtedly the most characteristic type produced by the diphtheria bacillus and frequently can be recognised with a considerable degree of certainty. It is the type which should always arrest attention in picking colonies. As the authors mention, the second and third types of colony are much less frequently encountered. This may be seen by analysis of the results obtained with thirty-nine different strains of diphtheria bacilli on their medium. Of the thirty-nine strains thirty-two gave the first type of colony, six the second type, while none of the third type were found in the series. One strain gave a smooth, white colony about 1.5 mm. in diameter with no tendency to blacken. The authors do not mention the occurrence of this type of colony which is apparently uncommon. Hofmann's bacillus and other diphtheroids of common occurrence in the nose and throat are capable of producing all three types of colony described as characteristic of the diphtheria bacillus. It has repeatedly been found that when a colony conforming to one of the above types has been picked it yields a growth not of diphtheria bacilli but of a diphtheroid. It is necessary to sub-culture the organisms on serum in order to make certain that it is a diphtheria bacillus, which has been picked, owing to the fact that the microscopical morphology of the diphtheria bacillus is not reproduced on the special medium and it cannot therefore be recognised by staining a film from one of the colonies. It should, however, be stated that Hofmann's bacillus and the other diphtheroids frequently produce colony forms which are not given by the diphtheria bacillus, and these do not cause confusion. The net result is that in picking colonies one should select those which conform to one of the types given by the diphtheria bacillus, ignoring the others. By doing this a considerable number of the diphtheria group other than the diphtheria bacillus will be picked, but at least none of the diphtheria bacilli will be lost.

With regard to the fourth claim, the only odour noticeable from the plates on which diphtheria bacilli are grown is the garlic-like odour of certain tellurides. It is produced by the diphtheroids which have black colonies just as readily as by the diphtheria bacillus and does not serve as a guide.

To turn now to the application of the method to the isolation of the diphtheria bacilli present in the serum cultures made during the mass swabbing. On the day that these cultures are made the requisite number of plates



of Allison and Ayling's medium to deal with them all are poured. They may be kept in the cold room over night and dried for a couple of hours on the next day before use. After the primary cultures have been incubated for 24 hours at 37° C., 1 c.c. of saline is added to each tube (a smaller amount being used when the growth is very light) and, after emulsifying the growth in the saline, films are made from the suspensions so obtained. The films are stained by one of the recognised methods of staining for diphtheria bacilli, the best and also the simplest being Loeffler's methylene blue. They are then examined microscopically for diphtheria bacilli.

No general rules can be given for the certain microscopical recognition of the diphtheria bacillus. It is a matter of considerable difficulty in many cases and facility of recognition can only be acquired by long and careful practice. In a given case it will, in all probability, be found that in some of the films from the cultures morphological diphtheria bacilli are present whereas in the majority of the films none can be seen. All the suspensions from the cultures, however, irrespective of whether diphtheria bacilli can be seen or not, should be inoculated heavily on the plates of the special medium and spread in the usual manner. Two or three loopfuls or more may be used for each plate.

The reason for plating all the suspensions is that Allison and Ayling's medium permits the ready isolation of diphtheria bacilli from suspensions of mixed organisms even when they are greatly outnumbered by these organisms and are present in such small numbers that they cannot be found in films from the suspensions. It may be argued that it is not worth while isolating the organisms from a carrier who gives such a light primary growth. Copeman, O'Brien, Eagleton and Glenny (1922) adopt the view that the risk from a carrier is proportional to the profuseness of the growth obtained from a swab on a serum slope. It should, however, be pointed out that even though the organisms are present in the nose and throat in considerable numbers it may well happen that the swab does not come in contact with a large number of them and consequently does not give a profuse growth. It must fall to every bacteriologist's lot to encounter bacteriologically established cases of diphtheria in which swabs yield little or no apparent growth of the diphtheria bacillus although it is abundantly present in the throat. As a matter of fact it is well known that carriers are notoriously intermittent in the profuseness of the growth which they yield, at one time giving a well-marked growth of the diphtheria bacillus, and at another no apparent growth at all. This intermittency is well shown by the experience of McCartney and Harvey (1928). It is consequently just as important to detect those carriers who show no apparent growth in the primary cultures on serum as those who provide a heavy growth.

Owing to the fact, already alluded to, that Allison and Ayling's medium suppresses a proportion of diphtheria bacilli, it is necessary, in order to ensure isolation of all the strains which are visible in the films from the primary cultures, to plate out the positive suspensions on an alternative medium.

Blood agar has been found very satisfactory for this purpose after a little practice in recognition of the various colony forms which the diphtheria bacillus may produce on it. Alternatively plates of Douglas's medium or Loeffler's serum may be employed according to choice.

After 24 hours' incubation at 37° C. it is possible to pick suspicious colonies from Allison and Ayling's medium. It is wise to re-incubate the plates for a further 24 hours in cases where no suspicious colonies have appeared. The sub-cultures should be made on Loeffler's serum. It will be found that in most cases the special medium will give a culture of diphtheria bacilli when these organisms were visible in the corresponding primary cultures. If however they have been suppressed by the special medium they may be isolated from the alternative medium. It is quite possible to ensure the isolation of diphtheria bacilli in every case from cultures, in which they are known to be present, by adopting this technique. Suspicious colonies should also be picked on to Loeffler's serum from the plates of the special medium spread with the suspensions of the primary cultures not known to contain diphtheria bacilli.

The serum slopes are incubated at 37° C. for 18 to 24 hours and are then filmed and stained to see if they have yielded a growth of diphtheria bacilli. All the morphological diphtheria bacilli are retained for fermentation and virulence tests, the Hofmann's bacilli and other diphtheroids being discarded. The fermentations of the suspected organisms are established by inoculating tubes containing glucose and saccharose in Hiss' serum water. The diphtheria bacillus always ferments glucose with the production of acid but never saccharose. The virulence test, which may be done concurrently with the fermentation test, is best carried out by the intracutaneous technique. Suspensions of the organisms to be tested are made from the serum slopes and standardised by opacity to contain about 500 million organisms per c.c. Two guinea-pigs each of about 250 gm. weight are used. One of the animals serves as a control and receives at the time of the test 500 units of antitoxin intracardially, or alternatively has been given 500 units intraperitoneally on the previous day. White-haired portions of the two animals are depilated and into corresponding sites in their skins 0.2 c.c. of the suspensions to be tested are inoculated intracutaneously. The injections should be at least one-half inch apart and up to ten tests may be made on each pair of animals. The unprotected animal should be given a dose of 125 units of antitoxin intraperitoneally 6 hours after the intracutaneous inoculations have been made. The readings are usually best made on the second or third day. The virulent organism is indicated by the fact that in the unprotected animal an erythematous patch appears at the site of the inoculation about 1 to 2 cm. in diameter and progresses to superficial necrosis in 2 or 3 days. In the protected animal no reaction occurs or at most a slight red papule which disappears in a few days.

## SUMMARY OF PROCEDURE.

A brief summary of the technique outlined above may now be inserted to make clear the different stages in the detection of the virulent carrier.

The first step is the removal of the diphtheria bacillus from the nose, throat or ear by means of a swab which is at once inoculated on Loeffler's serum. On the same day that the swabs are taken, plates of Allison and Ayling's medium are poured in readiness for the next day.

On the second day the growths on the slopes are emulsified in saline and examined microscopically for diphtheria bacilli. All the growths are sub-cultured on Allison and Ayling's medium, but those in which morphological diphtheria bacilli are present are sub-cultured on an alternative medium, such as blood agar, in addition.

On the third day suspicious colonies are picked on to serum slopes and the plates of the special medium are returned to the incubator for a further 24 hours' incubation.

On the fourth day the plates are examined once more to see if any further suspicious colonies have appeared. These are transferred to serum slopes as on the previous day. The growths from the serum slopes, inoculated the previous day, are examined for morphological diphtheria bacilli and these are tested for their fermentations and virulence.

By the seventh day at latest all the diphtheria bacilli present in the original swabs should be completely identified.

The number of individuals whom one person can deal with at a time, using the foregoing technique, is about a hundred. It is well to determine that the person carrying out the operation is not himself a carrier.

## DISCUSSION OF RESULTS WITH EXAMPLES.

The technique outlined above, with a few minor modifications, has been applied by me (1931) to the detection of diphtheria carriers among Dublin school children. The investigation required the isolation of diphtheria bacilli in as large a number of instances as possible from single nose and throat swabs taken from 1000 apparently healthy school children. 2000 swabs in all were therefore submitted to examination, but of these 300 were plated on Allison and Ayling's medium only, as it was not realised at first that the medium suppresses the diphtheria bacillus in some cases. A number of strains were lost on this account before the method of plating the primary serum growths, in which diphtheria bacilli were microscopically visible, on an alternative medium was adopted. Omitting consideration of the first 300 swabs, the results achieved by the technique described above with the remaining 1700 were as follows.

In films made from the primary serum growths morphological diphtheria

bacilli were visible in sixteen instances. They were isolated in pure culture by means of the special medium in fourteen instances, being suppressed in two cases. In these two cases they were isolated without great difficulty from blood agar plates, so that no strain was lost. Diphtheria bacilli were, however, isolated from twenty-three other primary cultures, in which they were not visible microscopically, by plating on the special medium. Assuming that the proportion of organisms suppressed by the medium remained the same for those which could not be seen in the primary cultures as for those that could, the number of organisms present, of which twenty-three were isolated, would be twenty-six. From this we may form an estimate of the efficiency of the method, the percentage efficiency being

$$\frac{(16 + 23)}{(16 + 26)} \times 100, \text{ i.e. } 93 \text{ per cent.}$$

In other words, there is a reasonable likelihood of isolating 90 per cent. of the diphtheria bacilli present in a series of swabs taken from the nose and throat. Although the numbers quoted in support of this contention are rather small, the series of swabs referred to above may be taken as representative of what is likely to be met with in practice. The number of colonies which grew on the special medium varied from only two or three up to a couple of hundred, showing that the numbers of diphtheria bacilli present in the original swabs varied from very few to quite large numbers. It should be mentioned that the above figures relate only to nose and throat swabs; the efficiency with ear swabs is liable to be less, owing to the increased difficulty of isolation caused by the frequent presence of spreading organisms.

Owing to the fact, already mentioned, that diphtheroids of various sorts are capable of producing colonies, identical in all respects with those of the diphtheria bacillus when grown on the special medium, and that they cannot be distinguished from one another microscopically in preparations from this medium, it is necessary to sub-culture more than one colony on to serum for every diphtheria bacillus isolated. The average number of colonies which must be picked in this way for each diphtheria bacillus isolated is five.

A final point of importance is that growing the diphtheria bacillus on the copper sulphate containing medium does not alter its virulence.

#### APPLICATIONS OF THE METHOD.

It will be seen from the foregoing that a technique is available which will enable one to detect the vast majority of carriers by examination of material removed from suspects at a single swabbing.

The detection includes determination of virulence which is, of course, all-important from the preventive point of view. The application of the method to the detection of carriers in the general population is indicated by me elsewhere (1931). The application of the method in a few other specific instances

may now be considered briefly. The cases it is proposed to deal with presume sporadic outbreaks of diphtheria in the following institutions:

- (a) a day school;
- (b) a small residential institution;
- (c) a large residential institution;
- (d) a general hospital.

To consider first the case of the day school. If it is a small school of young and consequently susceptible children, all should be swabbed and dealt with in accordance with the technique described above. The children may be Schick tested by an assistant at the time that the swabs are taken, in order to find the susceptibles preparatory to immunisation. As each child is finished he should be sent home and the school may be closed for four days until the isolation of the morphological diphtheria bacilli in pure culture is complete. The school may then be re-opened, those children who have been found morphological carriers being kept at home. These in due course are separated into virulent and non-virulent carriers, the latter being allowed to return to school, the former being retained and treated. It is advisable to swab the members of the families of virulent carriers in order to determine whether or not there are other carriers in the home.

If the school is too large for all the children to be swabbed, only those classes which have been subject to outbreaks of diphtheria may be dealt with or else the Schick test may be performed first to distinguish susceptibles from non-susceptibles, the latter being then swabbed as the most likely to harbour virulent diphtheria bacilli. Schick positive individuals may, however, also harbour virulent diphtheria bacilli and these have actually been isolated from them as shown by McGuire and Hitchens (1923) and Beattie and Herron (1926). Considering that 80 per cent. or more of adults are Schick negative in certain communities and that their immunity is due to contact with virulent diphtheria bacilli, without the occurrence of the disease in most cases, it is probable that many Schick positive individuals at one time or another are carriers of the diphtheria bacillus in their noses or throats. As to the duration of the carrier state or the numbers of diphtheria bacilli carried, in such instances, there is no information, and this aspect of the subject would seem to lend itself to much needed study by means of the technique described in this paper. The three Schick positive carriers detected by McGuire and Hitchens, among seven virulent carriers tested, apparently harboured considerable numbers of the organisms.

The proposition of Okell, Eagleton and O'Brien (1924), that Schick positive reactors never harbour virulent diphtheria bacilli, detectable by ordinary swab-culture methods, unless they are suffering from or are incubating diphtheria would seem to require further proof.

In dealing with a small residential institution, it is well to swab all the residents, but in large residential institutions, it may only be possible to swab the Schick negative reactors. If the cases of diphtheria, however, have

occurred in only one section of a large residential institution divided up into sections, then the best procedure would be to swab all the residents in that section. It should be noted that in dealing with residential institutions, isolation is desirable as soon as possible. In consequence those carriers who give a growth of morphological diphtheria bacilli, as judged by examination of films from the primary serum cultures, should be isolated at once, the others being isolated subsequently as their detection is completed.

Sporadic outbreaks of diphtheria in a general hospital are a fairly common cause of great inconvenience. The carriers are to be sought among the members of the staff of the hospital and the occupants of the wards in which the cases have occurred. It is well to remember in dealing with this type of outbreak, that the diphtheria bacillus sometimes infects wounds or similar lesions and may have to be sought in some such situation. As the numbers of individuals to be dealt with in this type of outbreak are not very large, as a rule, the method should be very readily applicable and should ensure an immediate arrest of an outbreak of diphtheria. As in the case of a residential institution, isolation of those carriers, who give a positive culture of morphological diphtheria bacilli in the primary serum growth, should be carried out at once, isolation of the others following in due course.

#### SUMMARY.

The technique of detecting diphtheria bacillus carriers has found little recognition in the literature of diphtheria.

A method of detecting diphtheria carriers by single swabbings is described.

The method is somewhat elaborate but is highly efficient.

The results which may be obtained with it are discussed.

Its application in certain specific cases is briefly considered.

#### REFERENCES.

- ALLISON, V. D. (1930). *Brit. J. Exper. Path.* **11**, 244.  
 ALLISON, V. D. and AYLING, T. H. (1929). *J. Path. Bact.* **32**, 299.  
 BACTERIOLOGICAL COMMITTEE OF THE MEDICAL RESEARCH COUNCIL (1923). *Diphtheria: its Bacteriology, Pathology and Immunology*.  
 BEATTIE, M. and HERRON, E. (1926). *Bull. Hyg.* (1927), **2**, 288 (Abstract).  
 COPEMAN, S. M., O'BRIEN, R. A., EAGLETON, A. J. and GLENNY, A. T. (1922). *Brit. J. Exper. Path.* **3**, 42.  
 MCCARTNEY, J. E. and HARVEY, W. C. (1928). *Proc. Roy. Soc. Med.* **21**, 845.  
 MCGUIRE, P. F. and HITCHENS, A. P. (1923). *J. Amer. Med. Assoc.* **80**, 665.  
 OKELL, C. C., EAGLETON, A. J. and O'BRIEN, R. A. (1924). *Lancet*, i, 800.  
 O'MEARA, R. A. Q. (1931). *Irish J. Med. Sci.* (March), 125.

# CONTRIBUTIONS TO THE EXPERIMENTAL STUDY OF EPIDEMIOLOGY. FURTHER OBSERVATIONS ON THE EFFECT OF VACCINATION ON HERD MORTALITY.

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(With two Graphs.)

IN the last memoir of this series (Greenwood, Topley and Wilson, 1931) we discussed at some length the modifications of the course of herd sickness effected by immunisation of entrants to the herds. Briefly, these were our conclusions: efficient artificial immunisation (the criteria of efficiency being the usual standardising methods of a laboratory) confers a considerable temporary advantage upon mice entering an infected herd. The risk of death during the period of herd life when the rate of mortality is especially high is greatly reduced, so that when one has the case of a herd or group temporarily exposed to special risks, the value of the expedient is very great. But no method of artificial immunisation we have tried will render the immunised animals impervious to the risks which their membership of the herd entails.

The experiments now to be described were designed to test two points. First of all we wished to study the course of herd mortality when recruitment was confined to immunised animals. Secondly we wished to compare the effects of immunisation by different routes.

*Exp. 1.* Taking the former problem first, if the reasoning of our last paper were just, we should expect that the rate of mortality in a herd recruited from immunised animals, or any function of the rate of mortality, would over a fair range of herd age be much more favourable than that of a herd recruited in the usual way from unsalted stock, but that mortality from the specific cause would not be extinguished. The experiment was inaugurated on 14. iii. 29 by bringing together 25 mice, each inoculated with 1000 *Bact. aertrycke*, and 100 normal mice. Three normal mice were added daily for 59 days. On 13. v. 29 the normal immigrants were replaced by 3 mice daily which had been vaccinated.

As in our previous experiments the mice were vaccinated by intraperitoneal injection of a killed formolised suspension of *Bact. aertrycke*, which contained both the "H" and the "O" antigens. Two doses of  $500 \times 10^6$  bacilli were given, with an interval of 1 week between the two doses. The interval between the administration of the second dose of vaccine and addition to the infected herd varied from 7 to 13 days. A certain number of mice were vaccinated each week, over and above those required for the daily additions; these were anaesthetised, bled to death from the jugular vein, and their sera tested for the presence of agglutinins. As it was known from previous experience (Topley 1929) that "H" agglutinins would almost invariably be

present, 45 out of 67 specimens of serum were put up against the "O" antigen of *Bact. aertrycke* only, so that it was possible to start at a low dilution of serum (1 in 5). The remaining 22 specimens were tested against both forms of antigen, starting at a dilution of 1 in 20. The results are given below.

Number of sera tested against "O" only	= 45
„ agglutinating "O" antigen	= 9 (20 per cent.)
„ tested against "O" and "H"	= 22
„ agglutinating "H" alone	= 20 (90.9 per cent.)
„ „ "O" „	= 0
„ „ both "O" and "H"	= 2 (9.1 per cent.)

Three mice continued to be added until 21. x. 29 from which date 1 vaccinated mouse was added daily until 29. vi. 30 when immigration ceased. On 29. viii. 30 the survivors were killed. Table I records the general experience of the normal mice which entered from 15. iii. 29 to 12. v. 29; Table II that of the vaccinated mice.

Table I. *Cage V. Normal mice, entrants 15. iii. 29-12. v. 29.*

No. of mice	...	...	...	...	...	...	177
No. who die	...	...	...	...	...	...	177
No. who die of specific ( <i>Bact. aertrycke</i> + N.E.) deaths	...	...	...	...	...	...	169
No. of mouse days exposed to risk	...	...	...	...	...	...	5870
Expectation of life at entry in days:							
(a) From specific deaths only	...	...	...	...	...	...	34.82
(b) From all deaths	...	...	...	...	...	...	32.66
Life table death-rate daily:							
(a) From specific deaths	...	...	...	...	...	...	0.0287
(b) From all deaths	...	...	...	...	...	...	0.0306
Expectation of life, limited to 60 days, at entry:							
(a) Specific deaths	...	...	...	...	...	...	27.00 $\pm$ 1.019
(b) All deaths	...	...	...	...	...	...	26.35 $\pm$ 1.003

Table II. *Cage V. Data used in the life tables.*

	Three mouse period	One mouse period
Period during which mice were entered	13. v. 29 to 20. x. 29	21. x. 29 to 29. vi. 30
No. of calendar days	161	252
Period covered by life table	13. v. 29 to 29. viii. 30	21. x. 29 to 29. viii. 30
No. of mice concerned	483	252
No. who die	482	242
No. who die of specific ( <i>Bact. aertrycke</i> + N.E.) deaths	396	220
No. surviving at the end of the period	1	10
No. of mouse days exposed to risk	19,207	10,461
Expectation of life at entry in days:		
(a) From specific deaths ( <i>Bact. aertrycke</i> + N.E.) only	47.04	48.16
(b) From all deaths	39.34	43.25
Life table death-rate daily:		
(a) From specific ( <i>Bact. aertrycke</i> + N.E.) only	0.0213	0.0208
(b) From all deaths	0.0254	0.0231
Expectation of life at entry limited to 60 days:		
(a) From specific ( <i>Bact. aertrycke</i> + N.E.) deaths	34.92 $\pm$ 0.684	36.38 $\pm$ 0.938
(b) From all deaths	32.11 $\pm$ 0.661	35.35 $\pm$ 0.932

For reasons discussed in earlier papers we think that the most useful standard of comparison is the expectation of life limited to 60 days and, although the estimated standard errors of this measure are only approxi-



mations, and we must remember that all survivors were killed, we think that a comparison is fair. In Table III and the graphs we show the probability of dying within the next 5 days for the various categories over the first 60 days of cage life.

From the nature of the experiment a strict control in time against unimmunised mice is not possible, the comparison with the normal additions *may* be unfair to the latter since their experience was during the epoch when the ratio of deliberately infected animals to all exposed to risk was large. But the general run of events is unmistakable. Putting the advantage of immunisation at its highest, *i.e.* by admitting the comparison of immunised and normals to be valid, we see that the immunised enjoyed in respect of limited expectation of life an advantage of the order of 50 per cent., but that during the time when all immigrants had been previously immunised the specific rate of mortality was still high and the vital statistics of the community very different from those of an uninfected herd. Restriction of immigrants to immunised animals did not bring the epidemic sickness to an end. This special experiment fully confirms the conclusions drawn from our earlier work. It may also be noticed that although reduction of the daily number of immigrants was associated with a decrease of the rate of mortality, the decrease was not large.

*Exp. 2.* We now pass to the experiment designed to test the relative advantages of intra-peritoneal and oral administration of vaccine.

Here again the vaccine used was a killed formolised suspension of *Bact. aertrycke* containing both the "O" and the "H" antigens. The P mice (see below) received two doses of  $500 \times 10^6$  bacilli; the O mice were given two doses of  $5000 \times 10^6$  bacilli introduced directly into the stomach by means of a fine ureteric catheter attached to a syringe. As before, a small number of extra mice were vaccinated each week, and the results of testing their sera for agglutinins are shown below.

Route	No. tested against "O" only (lowest dilution 1/5)		No. positive		
I.P.	14		4 (28.6 %)		
<i>Per os</i>	20		6 (30.0 %)		
	No. tested against "O" and "H" (lowest dilution 1/20)				
			"H" + only	"O" + only	"O" and "H"
I.P.	14		12	0	2
<i>Per os</i>	23		8	0	0

It appears therefore that intraperitoneal vaccination produced "H" agglutinins in every mouse tested, whereas the "O" form was only found in about one-third of the sera. *Per os* inoculation, on the other hand, did not produce "H" agglutinins nearly so frequently—only 8 of 23 mice gave positive reactions, and those mostly to low titre. As regards "O" agglutinins there was no significant difference between the effects of oral and intraperitoneal administration.

These observations are in general accord with those of Pijper and Dau (1930), who noted the development of "O," but not of "H" agglutinins in human subjects who had received typhoid vaccine by the mouth. Since, as

Table III. *Cage V. Probability of dying in the next 5 days.*

Cage age in days	Total deaths			Specific deaths		
	Vaccinated mice			Vaccinated mice		
	Normals entrants 15. iii. 29– 12. v. 29	Three mouse period 13. v. 29– 20. x. 29	One mouse period 21. x. 29– 29. vi. 30	Normals entrants 15. iii. 29– 12. v. 29	Three mouse period 13. v. 29– 20. x. 29	One mouse period 21. x. 29– 29. vi. 30
0	0-0057	0-0104	0-0079	0-0057	0-0104	0-0040
1	0	0-0104	0-0119	0	0-0083	0-0080
2	0-0057	0-0104	0-0119	0-0057	0-0063	0-0080
3	0-0170	0-0104	0-0159	0-0170	0-0063	0-0159
4	0-0341	0-0084	0-0120	0-0284	0-0042	0-0120
5	0-0511	0-0105	0-0160	0-0456	0-0063	0-0160
6	0-0739	0-0147	0-0201	0-0627	0-0126	0-0201
7	0-0800	0-0274	0-0321	0-0689	0-0232	0-0321
8	0-0983	0-0358	0-0364	0-0874	0-0316	0-0364
9	0-1118	0-0526	0-0445	0-1004	0-0464	0-0405
10	0-1138	0-0613	0-0488	0-0964	0-0530	0-0447
11	0-1288	0-0768	0-0533	0-1171	0-0665	0-0493
12	0-1615	0-0887	0-0498	0-1439	0-0804	0-0458
13	0-1731	0-1135	0-0672	0-1558	0-0966	0-0633
14	0-1722	0-1133	0-0678	0-1604	0-0961	0-0678
15	0-2095	0-1239	0-0769	0-2036	0-1043	0-0726
16	0-2324	0-1386	0-0909	0-2267	0-1072	0-0867
17	0-2444	0-1591	0-1092	0-2444	0-1183	0-1050
18	0-2558	0-1601	0-0991	0-2558	0-1177	0-0949
19	0-3200	0-1754	0-1227	0-3200	0-1254	0-1141
20	0-2991	0-2031	0-1343	0-2991	0-1401	0-1298
21	0-2844	0-1957	0-1381	0-2844	0-1373	0-1288
22	0-3235	0-1977	0-1422	0-3235	0-1396	0-1280
23	0-3542	0-2287	0-1650	0-3542	0-1731	0-1461
24	0-3529	0-2523	0-1606	0-3529	0-2047	0-1461
25	0-3780	0-2548	0-1604	0-3780	0-2149	0-1459
26	0-3974	0-2600	0-1934	0-3846	0-2211	0-1839
27	0-4203	0-2359	0-1886	0-4080	0-2069	0-1837
28	0-4032	0-2015	0-1976	0-3905	0-1806	0-1976
29	0-4182	0-2154	0-2469	0-4058	0-1898	0-2407
30	0-4706	0-2467	0-2675	0-4393	0-2277	0-2615
31	0-4468	0-2613	0-2534	0-4263	0-2264	0-2335
32	0-3750	0-2811	0-2676	0-3519	0-2376	0-2481
33	0-3514	0-2905	0-2687	0-3273	0-2374	0-2491
34	0-3125	0-3005	0-2541	0-2870	0-2449	0-2321
35	0-2593	0-2701	0-3130	0-2593	0-2104	0-2748
36	0-2308	0-2622	0-2936	0-2308	0-2254	0-2677
37	0-2000	0-2628	0-2788	0-2000	0-2359	0-2525
38	0-1667	0-2886	0-2857	0-1667	0-2586	0-2596
39	0-0909	0-2815	0-2967	0-0909	0-2469	0-2789
40	0-0500	0-2756	0-2278	0-0500	0-2385	0-2152
41	0-1000	0-3306	0-2857	0-1000	0-2963	0-2740
42	0-1000	0-3217	0-2933	0-1000	0-2779	0-2817
43	0-1500	0-3113	0-2429	0-1500	0-2705	0-2304
44	0-2000	0-2784	0-1875	0-2000	0-2510	0-1742
45	0-1579	0-3043	0-1967	0-1579	0-2858	0-1803
46	0-1111	0-2469	0-1636	0-1111	0-2268	0-1466
47	0-1111	0-2564	0-1509	0-1111	0-2462	0-1336
48	0-0588	0-2329	0-1509	0-0588	0-2192	0-1336
49	0-0625	0-2143	0-2115	0-0625	0-2003	0-1758
50	0-0625	0-2031	0-1837	0-0625	0-1889	0-1638
51	0-1875	0-1639	0-1304	0-1875	0-1490	0-1092
52	0-1875	0-1724	0-1111	0-1875	0-1576	0-0894
53	0-1875	0-1786	0-1333	0-1875	0-1607	0-1122
54	0-1333	0-2000	0-0732	0-1333	0-1826	0-0732
55	0-2000	0-1961	0-0500	0-2000	0-1786	0-0500
56	0-2308	0-2549	0-0750	0-2308	0-2387	0-0750
57	0-2308	0-2083	0-0750	0-2308	0-1911	0-0750
58	0-2308	0-2174	0-0513	0-2308	0-1957	0-0513
59	0-2308	0-1818	0-0804	0-2308	0-1591	0-0804
60	0-1667	0-1707	0-0804	0-1667	0-1477	0-0804

## CAGE V

PROBABILITY OF DYING IN THE NEXT FIVE DAYS

Normals entrants 15.3.29-12.5.29  
 Vaccinated mice, three mouse period 13.5.29-20.10.29  
 Vaccinated mice, one mouse period 21.10.29-29.6.30

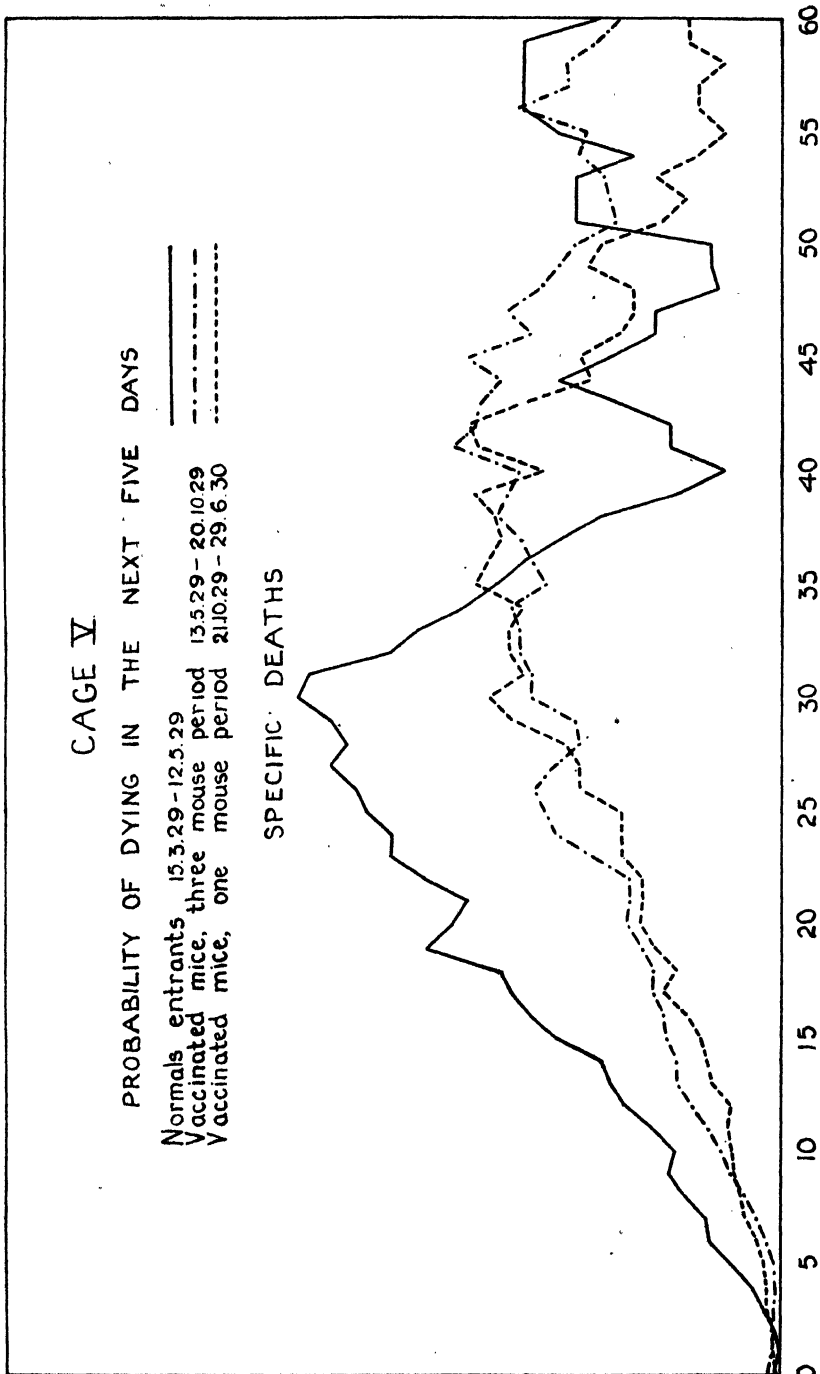
SPECIFIC DEATHS

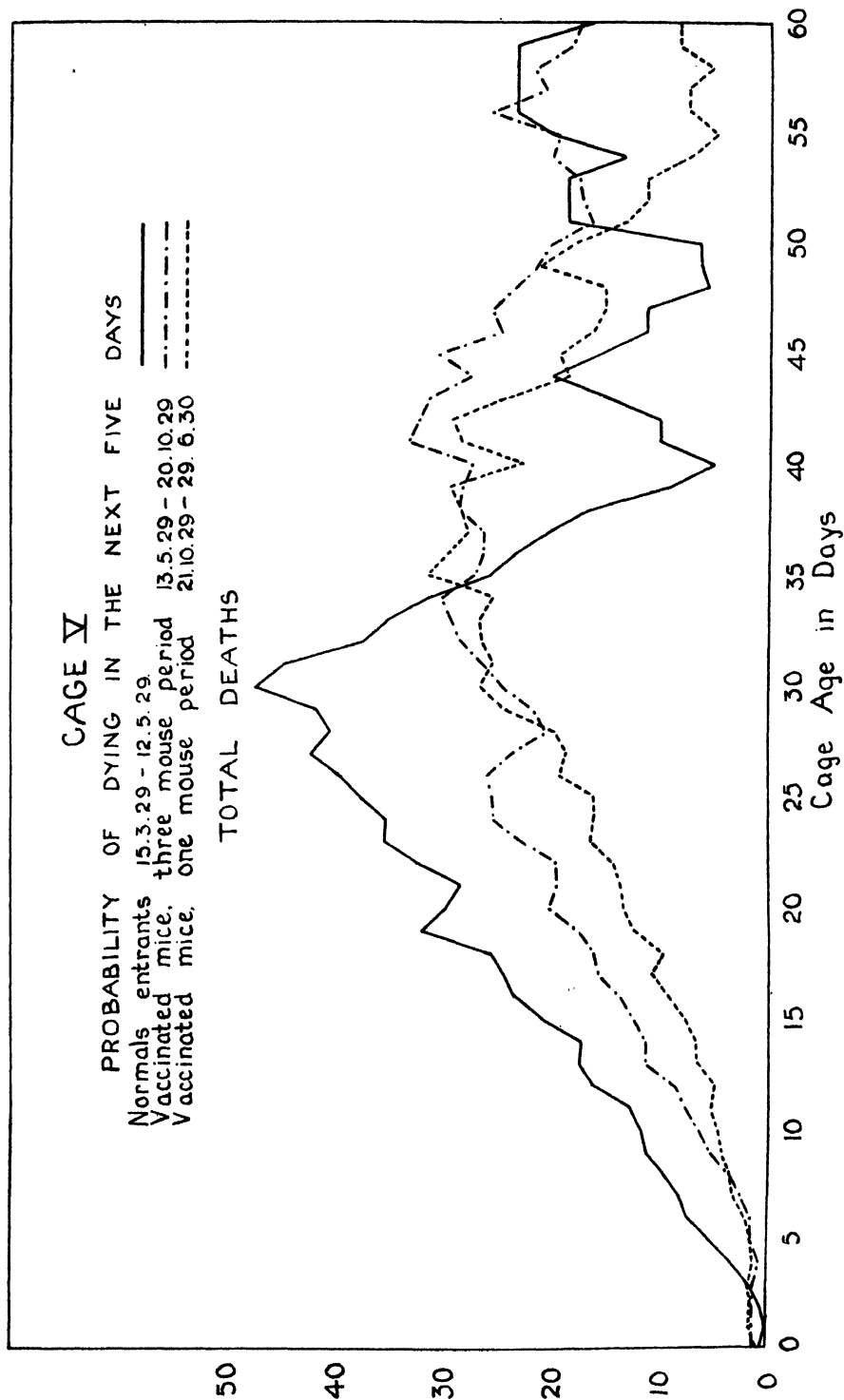
.50  
 .40  
 .30  
 .20  
 .10  
 0

60

Cage Age in Days

Graph I.





Graph II.

will be seen, the advantage of the mice vaccinated *per os* was but little inferior to that of those vaccinated by the intraperitoneal route, the results of this experiment afford additional evidence of the preponderating rôle of the "O" antibodies in immunity.

The experiment was inaugurated on 3. x. 29 by bringing together 25 mice each inoculated with 1000 *Bact. aertrycke* and 100 normals. Three normals were added daily down to 12. xii. 29. Thenceforward 2 normals were added daily and at weekly intervals three batches of 10 each were added, viz.:

O—mice vaccinated orally;

P—mice vaccinated intraperitoneally;

N—normal mice.

On 24. iv. 30 the last of the weekly batches was added.

On 22. vi. 30 the last daily addition was made.

On 23. vi. 30 all survivors were killed.

Table IV contains the summarised particulars. Table V contains a detailed comparison of the limited expectations of the several batches. Table VI compares, account being taken of estimated error of sampling, the limited expectations of life at various herd ages from day 0 to day 40.

Table IV.

Period covered	...	...	...	N	O	P	Added daily
				12. xii. 29– 23. vi. 30	12. xii. 29– 23. vi. 30	12. xii. 29– 23. vi. 30	9. xii. 29– 23. vi. 30
No. of calendar days	...	...	...	193	193	193	196
No. of mice concerned	...	...	...	200	200	200	283
No. who die of <i>Bact. aertrycke</i>	...	...	...	135	129	117	190
No. who die of N.E.	...	...	...	44	34	37	59
No. who die of nil found	...	...	...	11	9	9	7
No. surviving at end of period	...	...	...	10	28	37	27
No. of mouse days exposed to risk	...	...	...	7397	9137	10,064	10,804
Expectation of life at entry in days:							
(a) Unlimited from specific deaths only	...	...	...	40.41	55.24	65.02	44.65
(b) From all deaths	...	...	...	38.38	52.61	61.46	43.44
Limited to 60 days:							
(a) From specific deaths only	...	...	...	31.18	35.30	38.19	30.75
(b) From all deaths	...	...	...	30.16	34.45	37.32	30.23
Life table death-rates:							
(a) From specific deaths only	...	...	...	0.0247	0.0181	0.0154	0.0224
(b) From all deaths	...	...	...	0.0261	0.0190	0.0163	0.0230

It will be seen that both P and O mice have more favourable experiences than either N or Added Daily mice, over a considerable range of herd life. Take for instance the limited expectation at entrance (specific deaths), the difference between the P mice and the normals is 7.01 days or more than 22 per cent. of the expectation of the normals. The standard error of this difference is  $\pm 1.57$ , so that it is unlikely to be a mere random fluctuation. The advantage is consistently maintained to the 40th day of cage life, although we have then reached a stage where the ratio of the difference to its standard error is much smaller than before. The difference between the P mice and the best of the normals is 6.21 days with a standard error of 3.18. At later

ages the advantage of the vaccinated mice is negative or imperceptible. Throughout, the mice vaccinated *per os* have a smaller advantage than the mice vaccinated intraperitoneally.

Table V. *Expectation of life of each batch limited to 60 days.*  
*Specific deaths only.*

Date of entry	Added daily (against midday of the 7 days)	N	O	P
12. xii. 29	33.89	32.05	25.70	36.90
19. xii. 29	25.22	22.00	43.50	37.80
26. xii. 29	30.07	37.50	38.25	28.80
2. i. 30	27.82	26.55	37.98	43.76
9. i. 30	40.35	32.60	50.20	41.17
16. i. 30	26.96	30.10	25.65	38.70
23. i. 30	39.29	38.11	31.50	38.75
30. i. 30	33.43	29.62	28.15	37.70
6. ii. 30	37.72	42.00	45.10	47.90
13. ii. 30	35.69	31.50	43.85	41.69
20. ii. 30	26.96	42.24	38.25	42.80
27. ii. 30	28.08	34.61	35.15	31.45
6. iii. 30	34.96	31.05	36.12	40.40
13. iii. 30	27.11	29.30	38.45	43.50
20. iii. 30	31.00	27.20	28.70	38.78
27. iii. 30	31.43	22.50	25.40	38.53
3. iv. 30	27.29	34.60	40.75	34.17
10. iv. 30	29.90	33.00	28.46	42.10
17. iv. 30	23.11	20.00	36.15	28.35
24. iv. 30	20.07	25.60	26.00	37.00

Table VI. *Expectation of life limited to 60 days.*

Cage age in days	N	O	P	Added daily
		Specific deaths.		
0	31.18 $\pm$ 1.04	35.30 $\pm$ 1.22	38.19 $\pm$ 1.17	30.75 $\pm$ 0.93
10	22.94 $\pm$ 1.06	28.07 $\pm$ 1.23	31.22 $\pm$ 1.18	23.08 $\pm$ 0.95
20	18.34 $\pm$ 1.19	25.09 $\pm$ 1.36	26.67 $\pm$ 1.25	19.36 $\pm$ 1.07
30	23.56 $\pm$ 1.69	29.81 $\pm$ 1.71	30.27 $\pm$ 1.61	25.67 $\pm$ 1.49
40	39.17 $\pm$ 2.49	44.04 $\pm$ 2.23	45.38 $\pm$ 1.97	38.92 $\pm$ 2.03
50	39.79 $\pm$ 2.73	45.06 $\pm$ 2.37	47.97 $\pm$ 2.15	45.07 $\pm$ 2.32
60	40.19 $\pm$ 2.94	49.21 $\pm$ 2.63	49.69 $\pm$ 2.38	48.36 $\pm$ 2.49
70	42.40 $\pm$ 3.21	44.87 $\pm$ 2.73	49.52 $\pm$ 2.49	50.91 $\pm$ 2.63
80	42.12 $\pm$ 3.57	44.47 $\pm$ 2.92	48.90 $\pm$ 2.68	51.19 $\pm$ 2.89
90	46.81 $\pm$ 4.08	45.78 $\pm$ 3.26	47.38 $\pm$ 2.83	50.59 $\pm$ 3.11
		Total deaths.		
0	30.16 $\pm$ 1.02	34.45 $\pm$ 1.20	37.32 $\pm$ 1.15	30.23 $\pm$ 0.92
10	22.03 $\pm$ 1.04	27.28 $\pm$ 1.21	30.66 $\pm$ 1.17	22.85 $\pm$ 0.94
20	17.70 $\pm$ 1.17	24.70 $\pm$ 1.33	26.21 $\pm$ 1.23	19.00 $\pm$ 1.06
30	22.35 $\pm$ 1.66	29.58 $\pm$ 1.68	29.40 $\pm$ 1.49	25.09 $\pm$ 1.48
40	38.02 $\pm$ 2.44	43.64 $\pm$ 2.19	43.58 $\pm$ 1.95	38.26 $\pm$ 2.01
50	39.79 $\pm$ 2.68	44.23 $\pm$ 2.33	47.16 $\pm$ 2.12	45.07 $\pm$ 2.30
60	40.19 $\pm$ 2.89	47.83 $\pm$ 2.58	49.69 $\pm$ 2.37	48.36 $\pm$ 2.47
70	42.40 $\pm$ 3.15	43.13 $\pm$ 2.68	49.52 $\pm$ 2.46	50.91 $\pm$ 2.60
80	42.12 $\pm$ 3.50	43.52 $\pm$ 2.86	48.72 $\pm$ 2.64	51.19 $\pm$ 2.86
90	46.81 $\pm$ 4.00	44.47 $\pm$ 3.20	46.62 $\pm$ 2.79	50.59 $\pm$ 3.08

It will be seen, therefore, that these two experiments are fully accordant with those described in our last memoir and the conclusions therein drawn need no modification.

With regard to the other point at issue—the relative effectiveness of vaccination by the alimentary tract as compared with direct inoculation into

the tissues—the results are quite clear cut, and are in accord with those of many other observers. It is obviously possible to produce a significant degree of active immunisation by administering large doses of *Bact. aertrycke* vaccine by the mouth, although this route would appear to be slightly less effective than the intraperitoneal. There is no evidence that the immunity induced by oral administration differs in kind from that induced by inoculation into the tissues; had the former method of administration been followed by any local immunisation of the intestinal mucosa it would have been reasonable to expect the immunity produced to be appreciably greater than that following intraperitoneal administration.

We should desire to express our indebtedness to the Medical Research Council, who have provided a grant to cover the expenses of this investigation and the salary of one of us (J. W.).

#### REFERENCES.

- GREENWOOD, M., TOPLEY, W. W. C. and WILSON, J. (1931). *J. Hygiene*, **31**, 257.  
PIJPER, A. and DAU, H. (1930). *Brit. J. Exp. Path.* **11**, 112.  
TOPLEY, W. W. C. (1929). *Lancet*, *i*, 1337.

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# FURTHER OBSERVATIONS ON THE OCCURRENCE OF IODINE IN RELATION TO ENDEMIC GOITRE IN NEW ZEALAND AND ON IODINE METABOLISM.

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AND G. H. COX.

*(From the University of Otago, New Zealand.)*

(With a Map.)

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## I. INTRODUCTION.

IN a previous paper (Hercus and Roberts, 1927) an account was given of a comprehensive survey, which was carried out at Otago University, of the natural sources of iodine in New Zealand. This study also included observations on iodine metabolism, and on the effect of varying iodine environment on the iodine content of plant and animal products. More recently, we have carried out additional investigations with a view to further supplementing



our knowledge of various aspects of iodine distribution. The present paper deals with these later observations, which relate mainly to the iodine content of soils from parts of Australia and some of the Pacific Islands, and of articles of diet in New Zealand and the Islands, with the relations between the intake and excretion of iodine in human beings under various conditions, and with the distribution of iodine in the animal organism.

We have to acknowledge a grant from the New Zealand Department of Health which has enabled the analytical side of the research to be carried out; also a grant from the Sir John Roberts Endowment which assisted one of us (H. M. S. T.) in carrying out a section of the research.

We are also indebted to the Director of Canterbury Agricultural College for arranging to supply us with samples of soils and pastures; and to Dr K. C. Roberts for his co-operation in connection with the proof reading.

## II. SOME NOTES ON THE ANALYTICAL METHOD.

Most of the analyses which are here recorded were carried out by v. Fellenberg's method as described by Hercus and Roberts (1927). Not long ago the modification of Leitch and Henderson (1926) was introduced into our laboratory by Mr W. J. Blackie, M.Sc., and found to have considerable advantages in convenience. In this method the iodine is estimated by titration with a thiosulphate solution which ordinarily requires frequent standardisation. The use of amyl alcohol as described by Meyr and Kirschbaum (1928) to stabilise the thiosulphate solution was found to be highly satisfactory, the solution maintaining its strength unchanged for months.

In the course of our analyses we have frequently had occasion to observe the contamination of reagents and vessels by minute traces of iodine. Commercial brands of purified potassium hydroxide vary from sample to sample, some samples being iodine-free and others containing measurable amounts of iodine. Likewise various metal basins, otherwise suitable for combustions, were found to yield traces of iodine from their surfaces. Typical results are given in Table I.

Table I. *Showing amount of iodine obtained from various metal surfaces.*

Type	Microgram.
Nickel basin	0.5 (mean of 5 tests)
Cast-iron basin	1.6 (mean of 3 tests)
Iron ladle	1.4
Iron pot	0.9 (mean of 2 tests)
Monel basin (87 % nickel)	1.6
Copper basin	3.2
Platinum basin	Nil
Porcelain basin	2.4

In each case the results were obtained by evaporating potassium carbonate to dryness in the basin, combusting for a short time and analysing the carbonate for iodine. Since in actual estimations organic matter is present, which might be expected to inhibit this effect of alkali in extracting iodine from the metal, similar combustions were carried out in nickel, iron and monel basins

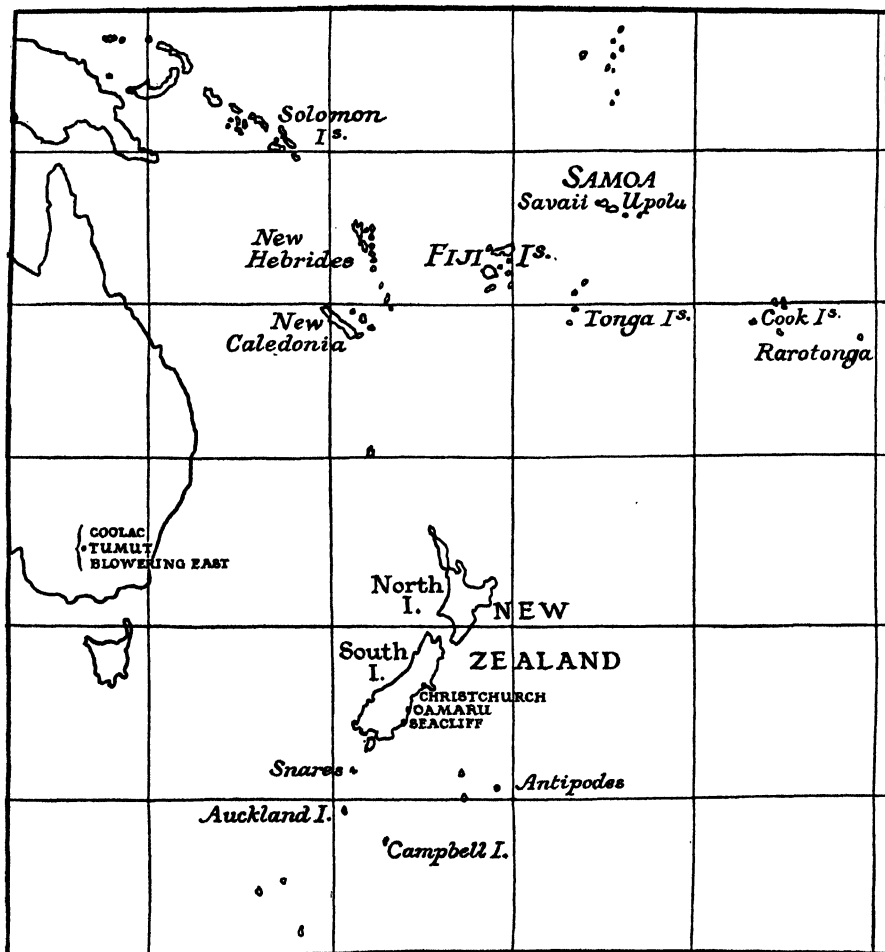
on a sample of fresh vegetable matter of known iodine content. It was found that the organic matter markedly reduced the amount of iodine given off by the metal, and that this source of error in the case of most of the nickel basins was negligible; nickel basins were, therefore, used throughout the work and any error so introduced may be assumed to be a constant one.

Another point which was investigated was the relation between the colour of the extract from combustion and the accuracy of the estimation. After a series of combustions with the same substance it was found that the most accurate results were obtained when the filtrate was straw colour.

### III. IODINE IN SOILS.

#### (1) *Iodine content of soils from districts in Australia and the Pacific Islands adjacent to New Zealand. (See Map.)*

As comparisons of iodine contents of soils from different localities are of very great interest whether from the agricultural, the medical, or the economic



point of view, we have taken the opportunity of supplementing our previous studies on the iodine content of New Zealand soils by obtaining specimens of soils from various Pacific Islands and from certain parts of Australia. The position of the various islands in relation to Australia and New Zealand is indicated in the Map. The iodine content of the soils and the geological formation from which they are derived are shown in Table II.

Table II. *Amount of iodine in soils of different origin and nature.*

Locality	Geological formation	Iodine in parts per 10,000,000	Goitre incidence %
Western Australia:			
Perth	Calcareous (?) sand	24	Nil
Kalgoorlie	Basic igneous rock	53	"
Bunbury	Loam	96	"
"	Sand	7	"
Freemantle	Calcareous (?) sand	24	"
Northam	Granite sand	8	"
New South Wales:			
Tumut	Slate?	—	—
Coolac	Slate?	15	—
Blowering East	Granite	38	—
Samoan Group:			
Savaii:			
Samatau	Basalt	351	Nil
Tuasivi	"	72	"
Sataua	"	720	"
Fagamalo	"	270	"
Satupaitea	"	360	"
Upolu:			
Vailele	Basalt	41	"
Apia	"	196	"
"	"	291	"
Cook Island Group:			
Rarotonga	Basalt	102	"
Solomon Islands:			
Inland village	Probably basalt or andesite	194	"
Coastal village		370	"
Tonga	Basalt	91	"
Antipodes at 400 ft.	"	144	—
Antipodes at 10 ft.	"	120	—
Auckland Island	"	120	—
Campbell Islands	"	103	—
Enderby Island	"	198	—
Goat Island	"	252	—
Snares Island	Granite	61	—
New Zealand:			
Canterbury:			
Christchurch	Gravel	25	55
"	Sandy	(mean of 8) 3	—
Otago:			
Oamaru	Gravel	98	—
Seacliff	Basaltic	69	10
Waitati	Phonolite	(mean of 11) 24	—
Stewart Island:			
Pegasus Bay	Granite	90	—
Halfmoon Bay	"	198	—

Table II again illustrates the higher concentration of iodine obtaining in soils derived from volcanic structures, as, for example, in the completely non-volcanic region of Samoa. Similar and even higher values have been obtained in certain volcanic regions in the North Island of New Zealand.

(2) *Iodine manuring and its effect on the iodine content of plants.*

Various investigators (Scharrer and Strobel, 1927; Orr, Kelly and Stuart, 1928) have shown that crops from soils treated with manures containing iodine show a higher iodine content than controls from unmanured plots. We have again taken up this question in order to find the nature of the increase which can be produced in the iodine content of grass by the use of iodides and also what results can be expected from the use of commercial manures.

In the first experiment a dressing of potassium iodide was given to six plots and different commercial manures were added to six other plots, the plots being then sown down with grass (15. x. 1929). The grass and soil from each plot was sampled on 5. xii. 1929, when the plots were showing a vigorous growth. Further samples of the maturer grass were collected on 24. iv. 1930. The results are shown in Table III. The figures in brackets represent the iodine content of the commercial manures.

Table III.

Plot	Manure	Total iodine in manure (gm.)	Iodine in soil (pts I <sub>2</sub> /10 <sup>7</sup> )	Iodine in grass Dec. 5th (pts I <sub>2</sub> /10 <sup>9</sup> )	Iodine in grass April 24th (pts I <sub>2</sub> /10 <sup>9</sup> )
1	Potassium iodide	·000765	51	150	210
2	"	·038250	67	160	220
3	"	·076500	89	260	210
4	"	·153000	93	270	330
5	"	·306000	110	340	250
6	"	·688500	131	—	390
7	20 grm. nitrate of soda (237/10 <sup>7</sup> )	·000474	51	150	200
8	20 grm. Seychelles guano (95/10 <sup>7</sup> )	·000190	42	130	190
9	20 grm. Nauru phosphate (28/10 <sup>7</sup> )	·000058	38	200	360
10	20 grm. superphosphate (152/10 <sup>7</sup> )	·000304	39	170	280
11	20 grm. special grass (76/10 <sup>7</sup> )	·000152	40	135	—
12	20 grm. blood and bone (1/10 <sup>7</sup> )	·000002	39	140	210

A study of Table III shows that in the first six plots the iodine content of the grasses reflects fairly definitely that of the dressing used. This is shown clearly in the young growth (Dec. 5th), while in the later growth (April 24th) the regular effect of increased iodine content in maturer vegetation is shown. The more even and apparently anomalous values obtained in the case of the later growth can be explained by assuming that the soils have a certain retentivity for iodine and that the excess iodine is largely washed out by the rain. Actually the soil was somewhat clayey and would have a rather low retentivity (Hercus and Roberts, 1927).

Further light was thrown on this point by testing for water-soluble iodine in the soils. For the first three plots the quantity was not measurable, while

in the later plots it reached only about a quarter of the total iodine content of the soil. Since the iodine content of these soils was raised considerably as compared with the control, it follows that even in the short time which had elapsed since manuring (about 7 weeks) much of the retained iodine had gone into organic combination. This is not difficult to understand on consideration of the ease with which oxidations are carried out by soil bacteria, for example nitrifying bacteria. The iodine is probably split off by bacteria, particularly in the presence of humic acids, from the readily oxidisable iodide and forms iodo-compounds with unsaturated organic matter in the soil.

The effect of the commercial manures is also interesting (Table III). It is seen that the iodine content of the pasture is not closely correlated either with the iodine content of the soil or with the relatively small iodine content of the manure. Obviously the availability of the soil iodine must vary from plot to plot. The iodine content of this grass compares favourably with that of the first six plots, and this seems to indicate that the manures exert their effect by rendering the soil iodine available, rather than by virtue of their own iodine. Table III shows that superphosphate is most effective in this regard. This is confirmed by the results of a similar experiment which was carried out for us at Lincoln Agricultural College, through the kindness of the Director. The details are given in Table IV. The pasture consisted of grasses and clover.

Table IV.

Plot	Manure	Iodine in soil (pts I <sub>2</sub> /10 <sup>7</sup> )	Iodine in pasture Aug. 1929 (pts I <sub>2</sub> /10 <sup>6</sup> )	Iodine in pasture Oct. 1929 (pts I <sub>2</sub> /10 <sup>6</sup> )
1	No manure for 8 years	32	220	194
2	Potash manure (30 %): 1 cwt. per acre per year for 6 years	36	248	380
3	Superphosphate (44 %): 1 cwt. per acre per year for 6 years	58	308	402
4	Lime: total of 5½ tons per acre over 6 years	22	253	378
5	Nitrate: 1 cwt. per acre per year for 4 years. Ammonium sulphate: 1 cwt. per acre per year for 2 years	20	220	—

In addition to artificial fertilisers dried seaweed and fish by-products represent sources of manure iodine. We have occasionally received samples of vegetables in which the iodine content has been increased by the use of seaweed manures. In other cases the results have been negative. This is attributed to differences in the variety of seaweed used and in the nature of the soil. The value of fish by-products as iodine manures depends upon the process of manufacture. A fish manure prepared by autoclaving refuse with steam under pressure was found to be useless as a source of iodine, most of the iodine having been volatilised.

## IV. IODINE IN FOODS.

(1) *Iodine content of edible products from districts in New Zealand and the Pacific Islands.*

Partly to complete our earlier survey of iodine in foods and partly for convenience in computing iodine intake on given diets we have made additional analyses of a number of articles of food as shown in Table V.

Table V. *The iodine content of foodstuffs and other substances.*

Substance	Source	Goitrous or Non- Goitrous	F. = Fresh D. = Dry	Iodine content in microgram. per kg.
<b>Cereals:</b>				
Oatmeal	Southland	G.	D.	21
Rice starch	?	?	D.	10
Raw wheat germ	Australia	—	—	40
White bread	Otago	G.	F.	6
<b>Root vegetables:</b>				
Beetroot (mean of 5) without skin	Canterbury	G.	F.	5.1
Beetroot (mean of 5) with skin	"	G.	F.	6.9
Carrot (mean of 8) without skin	"	G.	F.	4.2
Cucumber	Samoa	Non-G.	F.	2
Parsnip (mean of 6)	Canterbury	G.	F.	5.6
Potato	Samoa	Non-G.	F.	4
Taro	Solomon Islands	Non-G.	F.	2
Turnip (mean of 7)	Canterbury	G.	F.	5.7
<b>Leafy vegetables:</b>				
Brussels Sprouts	Otago	G.	F.	7.3
Cabbage (mean of 5)	Canterbury	G.	F.	6.1
Cauliflower	Otago	G.	F.	4
Leek (mean of 2)	Canterbury	G.	F.	8.4
Lettuce	Otago	G.	F.	6.9
Silver beet (mean of 16)	Canterbury	G.	F.	16.7
Spinach	Samoa	Non-G.	D.	255
"	Canterbury	G.	F.	6
Turnip top	"	G.	F.	26
<b>Fruits:</b>				
Apples (mean of 5) without peel or core	"	G.	F.	5
Apricots	"	G.	F.	4.8
Bananas	Samoa	Non-G.	D.	6
Cocoanut (edible portion)	"	Non-G.	F.	6
Egg fruit	"	Non-G.	F.	2
Lemon	"	Non-G.	F.	2
Mummy apple	"	Non-G.	F.	9
Orange	"	Non-G.	F.	2
Passion fruit	"	Non-G.	F.	2
Peanuts	"	Non-G.	F.	58
Pineapple	"	Non-G.	F.	2
Prunes	California	—	—	10
Raisins	"	—	—	32
Yeast	Otago	G.	D.	40
<b>Fresh water plants:</b>				
Watercress	"	G.	F.	31
<b>Grass:</b>				
Grass (mean of 11)	Samoa	Non-G.	D.	326
Grass	Canterbury	G.	D.	309
"	"	G.	F.	56
<b>Seaweeds:</b>				
Seaweed	Pacific Ocean	—	F.	5,830
Carrageen moss	Ireland	—	D.	315,000

Table V (*cont.*).

Substance	Source	Goitrous or Non- Goitrous	F. = Fresh D. = Dry	Iodine content in microgramm. per kg.
Plant oils:				
Cocoanut oil (edible)	Samoa	Non-G.	F.	6
Animal oils:				
Butter	Canterbury	G.	F.	6
"	Otago	G.	F.	12
Cod-liver oil (refined)	Scandinavia	?	F.	5,000
Shell fish:				
Oysters	New Zealand	—	F.	809
Animal products:				
Eggs	Canterbury	G.	F.	52
"	Otago	G.	F.	58
"	Samoa	Non-G.	F.	394
Cow's milk	"	Non-G.	F.	11
"	Canterbury	G.	F.	7
"	Otago	G.	F.	6
Cheese	"	G.	F.	31
Cream	"	G.	F.	6
Casein	New Zealand	—	D.	156
Lactose	"	—	D.	10
Meat:				
Tripe	Otago	G.	F.	5
Meatmeal	"	G.	D.	158
Miscellaneous:				
Marmalade (Californian oranges)	California	—	F.	10
Jam (plum)	Otago	G.	F.	12
Honey	"	G.	F.	18
Non-edible substances:				
Oyster shell	Otago	G.	D.	38,400
Sark	India	—	—	125

Carrageen, cod-liver oil and oysters, being marine products, are particularly rich sources of iodine. It is unfortunate that the price of fish in New Zealand compares badly with that of meat, with the result that fish is by no means a staple article of diet. Also in many districts such as the endemic area of South Westland, transport difficulties are so great that fish is practically unobtainable. Among the land products given in the table Samoan eggs are noteworthy, having an iodine content of 394 microgramm. per kg.

(2) *Iodine content of New Zealand foods as compared  
with those of Samoa.*

We have already recorded the results of a comparative study of the iodine content of foodstuffs in goitrous and non-goitrous districts in New Zealand. It was decided to extend this investigation to the goitre-free island of Samoa for purposes of comparison with more or less endemic districts in New Zealand, such as Canterbury and Otago. Owing to transport difficulties the analytical material was narrowed down to grass, eggs and milk. The findings are shown in Table VI.

Table VI shows clearly the marked difference between Samoa and New Zealand as iodine environments, and is thus further evidence for the relation between goitre incidence and iodine deficiency.

Table VI.

District	Goitre incidence (%)	Foodstuffs iodine in 10 <sup>9</sup>			
		Soils: iodine in 10 <sup>7</sup>	Grass: iodine in 10 <sup>9</sup>	Eggs	Milk
Samoa	Nil	285 (mean of 9)	326 (mean of 11)	394 (mean of 12)	11 (mean of 6)
Otago	19	64 (mean of 12)	68 (mean of 13)	58 (mean of 12)	8 (mean of 9)
Canterbury	64	25 (mean of 9)	67 (mean of 6)	41 (mean of 12)	6 (mean of 9)

(3) *Dietaries of institutions in relation to daily iodine intake.*

An investigation of the dietaries of fifteen residential institutions was carried out by one of us (H. M. S. T.) with a view to ascertaining to what extent the iodine content of the daily menu conformed to the accepted daily requirement of iodine. The fifteen institutions included four preparatory school hostels, seven secondary school hostels, two university residential colleges, one nurses' home and one mental hospital, the total number of residents being 1900. From data received from these institutions was compiled Table VI A, which shows the average diet for all the institutions and the corresponding iodine intake, exclusive of fish and iodised salt, which will be considered later.

Table VI A.

Food	Weight in grm.	I <sub>2</sub> intake for non-goitrous area		I <sub>2</sub> intake for goitrous area	
		Iodine in $\gamma$ /kg. (Hercus and Roberts, 1927)	Total I <sub>2</sub> in $\gamma$	Iodine in $\gamma$ /kg. (Hercus and Roberts, 1927)	Total I <sub>2</sub> in $\gamma$
Meat	168	16	2.4	9.5	1.4
Eggs	20	137	2.7	56	1.1
Butter	327	12	3.9	8	2.6
Milk (c.c.)	293	25	7.3	15	4.4
White cereals	220	3	0.66	2	0.44
Whole cereals	64	40	2.5	20	1.2
Fresh fruit	100	5	0.5	3	0.3
Dried fruit	40	20	0.8	12	0.5
Root vegetables	200	9	1.8	6	1.2
Leafy vegetables	20	27	0.5	20	0.4
Water (in c.c.)	1000	2	2.0	1	1.0
Total iodine intake		—	25.06	—	14.5

The iodine intake on this diet is too close to the minimum requirement (according to Orr and Leitch (1929) 15 microgram. for an adult, 50 microgram. for a child) to be suitable for individuals resident in a district where goitre is endemic. However, it was found that on an average the institutions investigated used fish about once a week, though four neglected the use of fish altogether. In a total of twenty-eight meals having fish as a basis, fresh fish (generally proper) was employed in nineteen, tinned salmon in six, sardines in two, and smoked cod in one. The effect on average daily iodine intake of having a meal of fish once a week is shown in Table VI B.



Table VI B.

Kind of fish	I <sub>2</sub> in γ/kg.	One serving	I <sub>2</sub> content	Increase in average daily I <sub>2</sub> intake by use once weekly
Fresh fish (Grouper)	72	100 grm.	7.2	1.0
Salmon	750	50 "	37.5	5.3
Smoked fish (blue cod)	812	90 "	73	10.4
Sardines	360	25-50 "	9-18	1.3-2.6
Oysters	880	1 oyster	20	3.0

It is clear that the use of salmon, blue cod, or oysters twice a week in conjunction with the diet shown in Table VI A would produce an important augmentation of the daily iodine intake. As to the cooking of the fish, our experiments have shown that there is no loss of iodine by baking, steaming or frying, though there is an appreciable loss on boiling, if the cooking water is discarded.

Reference to Table VI A shows that milk is an important item in supplying iodine, a pint of milk containing from 7 to 11.6 microgm. The standard for consumption of milk from the point of view of calcium and phosphorus requirement is generally given as  $1\frac{1}{2}$  to 2 pints daily for a child and 1 pint daily for an adult. The average consumption in the institutions considered was 0.63 pints per head daily, all being below the standard, while three institutions used less than half a pint per head daily. These latter were all school hostels!

Finally, it was found that while all fifteen institutions used iodised salt at table, only eight were using it for cooking purposes as well. Investigation has shown that table salt represents about 16 per cent. of the total salt used, and that the use of iodised salt containing 1 part of iodine in 250,000 solely at table raises the daily intake by only about 5 microgm. It is obvious that to obtain the full effects of iodised salt it should be used both for table and cooking purposes, and cooking waters in preference to being discarded should be used in the preparation of soups, sauces, etc.

## V. IODINE METABOLISM IN MAN.

At the present time our knowledge of iodine metabolism is extremely fragmentary. Little is known of the respective rôles of inorganic and organic iodine in nutrition, or of the relations between blood iodine, tissue iodine and thyroid iodine. Even the fundamental question of the relation between intake and excretion of iodine has received scant attention, the only experiments being one by McClendon and Hathaway (1923) and two by v. Fellenberg (1923-6). We have now carried out three studies on this subject as described below, with rather interesting results. Experiments on the effect of fasting on iodine excretion, on the iodine content of human milk, and on the effect of iodized salt on iodine excretion are also described.

(1) *Relations between intake and excretion of iodine on diets low in iodine and on normal diets.*

In the first study three women students, whose ages were 24, 19 and 21 years, and whose weights were 11 st. 8 lb., 10 st. 10 lb. and 9 st. 7 lb. respectively, agreed to undertake a quantitative investigation of 10 days' duration. The students aged 24 and 19, who will be referred to as students A. and B., had simple goitres of medium size of some years' duration—the third student, C., had an apparently normal thyroid gland. Throughout the investigation the students carried out their usual routine of classes and social activities.

The diet adopted is detailed in the Appendix, Diet A.

Each day each item of the diet was accurately weighed and the iodine content was determined. The daily iodine intake of the food and water will be seen to be approximately 8 microgm. After the diet had been in use for 4 days, in order to give an opportunity for the body to adjust itself to the low iodine intake, the total daily iodine excretion in urine and faeces was determined for each subject for a period of 6 days. The intake through the respiratory tract and the excretion by way of mucous discharge and perspiration have been neglected, being unimportant.

The results are given in Table VII.

Table VII.

Day of experiment	Daily iodine intake in microgm. Students			Daily iodine excretion in microgm. Students			Iodine balance in microgm. Students		
	A.	B.	C.	A.	B.	C.	A.	B.	C.
	Preliminary period			Preliminary period			Preliminary period		
1-4	8	8	8						
5	8	8	8	35.8	38.0	51.0	-27.8	-30.0	-43.0
6	8	8	8	31.9	33.7	50.0	-23.9	-25.7	-42.0
7	8	8	8	35.0	29.1	48.0	-27.0	-21.1	-40.0
8	8	8	8	54.0	33.8	27.6	-46.0	-25.8	-19.6
9	8	8	8	35.4	60.0	30.0	-27.4	-52.0	-22.0
10	8	8	8	83.2	29.3	27.2	-75.2	-21.3	-19.2
Total for 6 days	48	48	48	275.3	223.9	233.8	-227.3	-175.9	-185.8

If excretion values in Table VII, differing greatly from the average of all values, are rejected it is found that the tendency is for the excretion to approximate to some 34 microgm. per day, thus giving rise to an average balance of about -26 microgm. per day. The excretion corresponds to that associated with the normal diet of the students before the beginning of the experiment, and it is clear from the table that, even at the end of 10 days, the iodine of the tissues is still being mobilised, and the small intake has not yet led to the inevitable lower equilibrium. Only in the case of student C. do we observe the first signs of adjustment to the lower level. Such a lag can be explained by consideration of the small ratio which the daily intake of iodine bears to the total bodily iodine, especially as compared with the corresponding ratio for a more typical food element such as nitrogen. It would

seem that adjustments of iodine balance are mainly passive; as is to be expected, for if iodine excretion were under active control, iodine deficiency would be a rarity.

After a period of 3 weeks and 3 days had elapsed, a further study was carried out on a diet relatively rich in iodine-containing food, the total iodine content being 45 microgram. The details of the diet are given in the Appendix, Diet *B*. After the usual 4 days on the diet, estimations of iodine excretion through the urine and faeces were determined for a further period of 6 days. The results are given in Table VIII.

Table VIII.

Day of experiment	Daily iodine intake in microgram. Students			Daily iodine excretion in microgram. Students			Iodine balance in microgram. Students		
	A.	B.	C.	A.	B.	C.	A.	B.	C.
1-4	45	45	45	Preliminary period			Preliminary period		
5	45	45	45	34.4	32.0	40.0	+10.6	+13.0	+5.0
6	45	45	45	29.2	136.0	40.0	+15.8	-91.0	+5.0
7	45	45	45	30.2	78.6	45.0	+14.8	-33.6	0
8	45	45	45	32.4	56.0	36.0	+12.6	-11.0	+9.0
9	45	45	45	30.1	40.0	34.0	+14.9	+5.0	+11.0
10	45	45	45	29.6	21.4	28.2	+15.4	+23.6	+16.8
Total for 6 days	270	270	270	185.9	364.0	223.2	+84.1	-94.0	+46.8

In this table a normal excretion is found to be about 37 or 33 microgram. higher than the normal excretion for the low iodine diet of Table VII. The figures in column B. on the sixth and seventh days are obviously anomalous, being due to effects other than dietic (excitement, menstruation, etc.). There is a definite tendency in all three cases for storage to take place towards the end of the period, the tissues being perhaps slightly depleted as a result of the earlier experiment.

The third study was carried out on two members of the teaching staff of the Home Science School in conjunction with a study as to the intake and output of calcium. The subjects will be referred to as B. and N. Both were free from goitre. Their respective weights were 143 and 156 lb. and their ages 35 and 26 years.

The diet was one based upon an enquiry by Storms and Todhunter (1928) into the exact content of some of the diets of 710 families comprising 13,099 meals. This enquiry included data from over seventy different occupations from all strata of society scattered throughout New Zealand. The diet selected, details of which are given in the Appendix, Diet *C*, was constructed to represent, as far as possible, an average of these dietaries. Diets which were of apparently identical composition appeared in a number of those collected.

Before the experiment was commenced, observations as to intake and excretion were carried out for a fortnight on a well-balanced diet to which iodised salt was added in the usual proportions. Exact weighings were not

carried out with this diet, but qualitative data are given in the Appendix, Diet *D*. The diet was estimated to contain 45 microgm. of iodine. The daily excretion for B. averaged 34 microgm. in urine and 7 microgm. in the faeces, which gave a positive iodine balance of 4. The daily excretion of N. averaged 28 microgm. in the urine and 10 microgm. in the faeces, a positive balance of 7 microgm.

Diet *C* was now adopted on an accurate quantitative basis. For the first 4 days of the experiment the iodine excretion was not estimated. Exact data as to intake and excretion were now obtained by analysis for 6 days.

The results are given in Table IX.

Table IX.

Day of experiment	Daily iodine intake in microgm. Subjects		Daily iodine excretion in microgm. Subjects						Iodine balance in microgm. Subjects	
			B.			N.				
	B.	N.	Urine	Faeces	Total	Urine	Faeces	Total	B.	N.
	Preliminary period									
1-4	24	24							—	—
5	24	24	19.4	2.8	22.2	14.0	4.5	18.5	+1.8	+5.5
6	24	24	17.0	—	17.0	7.3	2.2	9.5	+7.0	+14.5
7	24	24	11.6	5.0	16.6	5.2	1.2	6.4	+7.4	+17.6
8	24	24	13.2	3.4	16.6	8.5	1.5	10.0	+7.4	+14.0
9	24	24	12.0	3.7	15.7	11.7	2.6	14.3	+8.3	+9.7
10	24	24	13.0	5.0	18.3	13.4	—	13.4	+5.7	+10.6
Total	—	—	—	—	—	—	—	—	+37.6	+66.9

In this experiment we might be led, on the basis of the previous results, to expect a balance of - 10 microgm. on such a change of diet. Actually there is a slight but definite increase of storage. The reason for this remarkable effect must be sought among the items of the diets (Appendix). Comparing Diets *C* and *D* we observe that, in *C*, *meat* has been substituted for the fish and eggs of *D*. Hence it would appear that iodised salt is much more effective in promoting storage when associated with a meat diet than when associated with a fish diet.

## (2) *Effect of fasting on iodine excretion.*

Two women students from the goitre-free district of Taranaki, aged 20 and 21 years and weighing 12 st. 13 lb. and 12 st. 5 lb. respectively, were anxious to undergo a period of fasting in order to reduce weight. These students will be referred to as Students 1 and 2. It was decided to carry out an iodine balance experiment during the fasting period. The girls were examined and found to be perfectly normal well-built girls. Their health was excellent, and they both engaged in strenuous athletics—hockey and tennis.

The diet on the day preceding the fast was accurately determined and the iodine content was estimated. A 5-day fast then followed, when 700 c.c. of water constituted the total intake of food. The total urine excreted throughout the fast period was collected and the iodine content determined. During

this period the students attended classes and carried on their usual activities, with the exception of the substitution of walking for all strenuous exercises.

On the day following the fast a very light diet was taken. It should be noted that the diet on the day preceding the fast included 60 grm. of salmon. The detailed constituents of the diet on this day were: meat, 62 grm.; salmon, 60 grm.; turnip, 90 grm.; potatoes, 120 grm.; plums, 200 grm.; apples, 117 grm.; figs, 28 grm.; dates, 35 grm.; maizena, 70 grm.; rice, 30 grm.; white bread toast, 150 grm.; cakes, 38 grm.; butter, 9 grm.; and water, 500 c.c. The energy value was approximately 2000 calories and the iodine content 50–60 microgrm.

On the day subsequent to the fast the diet contained: soup, 150 grm.; potatoes, 75 grm.; carrots, 71 grm.; white bread toast, 64 grm.; maizena, 68 grm.; butter, 15 grm.; and water, 550 c.c., the energy value being approximately 700 calories and the iodine content less than 10 microgrm.

The results of the experiment are given in Table X.

Table X.

Day of experiment	Daily iodine intake in microgrm.		Daily iodine excretion in microgrm.		Iodine balance in microgrm.	
	Student 1	Student 2	Student 1	Student 2	Student 1	Student 2
1 (preliminary)	50–60	50–60	69.0	62.0	–9.19	–2.12
2 (fast)	1.3	1.3	23.5	22.0	–22.2	–20.7
3 (fast)	1.3	1.3	29.4	17.8	–28.1	–16.5
4 (fast)	1.3	1.3	9.0	10.6	–7.7	–9.3
5 (fast)	1.3	1.3	7.6	9.2	–6.3	–7.9
6 (fast)	1.3	1.3	16.0	12.6	–14.7	–11.3
7	10.0	10.0	8.0	14.6	—	—

It will be seen that on the day preceding the fast the iodine excretion of the urine was at the relatively high level of 69 and 62 microgrm. On the first day of fasting the iodine excretion fell suddenly to 23 and 22 microgrm. respectively, and continued to fall with minor fluctuations. Student 1 lost a total of 79 microgrm. and Student 2 65.7 during the course of the fast. The weight of Student 1 was reduced by 10 lb. and that of Student 2 by 8 lb.

### (3) *Iodine excretion in human milk and urine.*

In a previous paper (Hercus and Roberts, 1927) a number of analyses are recorded showing the relationship between the iodine excretion in the milk and urine of goitrous and non-goitrous women.

It was shown that the iodine content of the milk is high immediately after parturition, and during the subsequent week falls rapidly to lower levels which are lower in goitrous subjects than in the non-goitrous.

Some further data on this subject are recorded in Table XI. The majority of the specimens were obtained from a maternity hospital in a non-endemic area. The mothers were unmarried girls of an average age of 19 years. The milk was expressed from the breasts on and after the third week after parturition.

Table XI.

Subject		Milk		Urine		Blood
Goitrous area	Non-goitrous area	Iodine in microgm. per litre	Iodine in microgm. per 24 hours	Iodine in microgm. per litre	Iodine in microgm. per 24 hours	Iodine in microgm. per kg.
C.	—	7.0	3.3	40	73	—
R.	—	10.7	9.6	—	—	—
	N.	24.0	19.0	90	54	105
	M. <sup>1</sup>	9.0	10.0	12	17	—
	M. <sup>2</sup>	12.0	13.0	22	27	78
	F.	16.7	10.0	14	11	79
	D.	8.0	10.0	6	10	52
	L.	30.0	23.0	22	31	—

Considerable difficulty was experienced in obtaining specimens for analysis, and the number of cases from endemic areas is too small to constitute a fair sample for statistical purposes. It will be seen, however, that the amount of iodine secreted in the milk in 24 hours averages 6.4 microgm. in the endemic areas, as compared with the average of 14 microgm. in the non-endemic area.

By the addition of iodised salt to the diet of one mother suffering from goitre and resident in an endemic area it was found possible to raise the iodine content of the milk in the secretion of 24 hours from 12 to 25 microgm. A definite reduction in the size of the goitre was also observed during a 6-month lactation period.

#### (4) *Effect of iodised salt on iodine excretion.*

Previous studies recorded in this *Journal* of the effect on urinary excretion of the addition of potassium iodide have been extended. The amount of iodine in 24-hour specimens from groups of twelve individuals at different age periods has been determined before and after the use of an iodised salt containing 0.7 parts of KI per 250,000 parts of salt. Specimens were obtained from adults in the Christchurch Mental Hospital and from the following Dunedin Institutions: two university women's colleges, a girls' school, and an orphanage. The ages of the subjects from whom the specimens were obtained were 20–40 years in the case of the mental hospital, 18–21 years in the university colleges, 12–14 years in the girls' school, and 2–8 years in the orphanage. Varying periods of time were allowed to elapse between the collection of these specimens; thus in the mental hospital iodised salt had been in constant use for 12 months, in the university colleges 8 and 12 weeks, in the girls' school 15 weeks, and in the orphanage 14 weeks.

The amount of salt consumed per day was estimated at 7.5 gm. This figure was based on the average consumption of 172 persons living in two residential colleges over a period of 6 days when the salt used for cooking purposes, on the table and any disused salt was accurately weighed.

It is evident, therefore, that each individual had his normal daily iodine intake supplemented by approximately 16 microgm. The average daily intake of iodine in the food was estimated to be approximately 20–30 microgm.

The results are given in Table XII.

Table XII.

Institution	Period iodised salt in use	Excretion of iodine in microgm. per 24 hrs.		Increase in microgm.	Percentage increase
		Before use of iodised salt	After use of iodised salt		
Mental hospital	12 months				
Goitrous average	—	20.0	37.0	17.0	85
Non-goitrous average	—	36.0	59.0	23.0	64
Women's college X	8 weeks				
Goitrous average	—	16.0	25.2	9.2	58
Non-goitrous average	—	25.6	29.1	3.5	14
Women's college Y	12 weeks				
Goitrous average	—	18.3	25.0	6.7	37
Non-goitrous average	—	24.3	29.4	5.1	21
Girls' school	15 weeks				
Goitrous average	—	11.5	18.5	7.0	61
Non-goitrous average	—	14.1	20.0	5.9	42
Orphanage	14 weeks				
Goitrous average	—	19.3	24.1	4.8	25
Non-goitrous average	—	16.5	26.3	9.8	59

It will be seen that in each group there is a definite increase in the iodine excretion varying from 14 to 85 per cent.

It is to be noted that among adults the iodised salt has raised the excretion in each case to the non-goitrous normal, fulfilling its purpose with remarkable accuracy. Among the girls, where the difference in total iodine between goitrous and non-goitrous excretions is much less than in adults, it has the effect of raising the excretion above normal.

(5) *Observations on the iodine content of blood, excreta and thyroid gland in different types of goitre occurring in New Zealand.*

Tables XIII (A-D) give the results of iodine estimations of the blood, urine and faeces of a number of patients treated for goitre in the Dunedin Public

Table XIII A. *Simple colloid goitre.*

Patient	No.	Sex	Age	Basal metabolic rate (mean value)	Iodine content of blood (microgm. per kg.)	Iodine excreted in urine (microgm. per 24 hrs.)	Iodine excreted in faeces (microgm. per 24 hrs.)	Total iodine excreted (microgm. per 24 hrs.)	Observations on iodine treatment, if any, and iodine content of gland in cases of operation (dried gland)
H. A.*	1	M.	21	+20	133	98	13	111	Iodised salt. Wt. of gland 301 grm. Iodine content 0.11 mg./grm.
O'G. J.	2	F.	28	- 6	400	15	5	20	Iodine content of gland 0.06 mg./grm.
B. B.*	3	M.	15	+10	173	22	—	—	Thyroid extract 1 grain thrice daily
R. S.	4	F.	36	+13	150	—	—	—	—
R. E.*	5	F.	38	+20	357	—	—	—	Iodised salt tincture 1 minim T.D.S.
E. A.	6	F.	19	- 7	57	19	23	41	—
S. A.*	7	M.	21	+20	405	319	112	431	Mis. pot. iod. 10 grain T.D.S. Thyroid extract 1 grain T.D.S.
R. R.	8	F.	27	+ 3	56	19	4	23	—
L. M.	9	M.	20	+ 6	67	31	—	—	—
H. W.	10	M.	24	+ 7	110	48	22	70	—

\* Indicates iodine treatment.

Table XIII B. *Simple nodular goitre.*

Patient	No.	Sex	Age	Basal meta- bolic rate (mean value)	Iodine content of blood (micro- gram. per kg.)	Iodine excreted in urine (micro- gram. per 24 hrs.)	Iodine excreted in faeces (micro- gram. per 24 hrs.)	Total iodine excreted per 24 hrs. (micro- gram.)	Observations on iodine treatment, if any, and iodine content of gland in cases of operation (dried gland)
O'G. V.	1	F.	34	+17	169	—	—	—	—
McK. I.	2	F.	44	+ 9	—	—	—	—	Iodine content of normal tissue 0.23 mg./gram. of adenoma 0.20 mg./gram.
H. G.	3	F.	23	+ 7	149	31	11	42	—
M. H.	4	F.	45	+30	—	—	—	—	Gland weighed 96 gram. A small nodule contained 0.16 mg./gram.; large nodule 0.14 mg./gram.
L. J.	5	F.	40	- 3	294	46	12	58	—
K. L.	6	F.	30	+ 7	50	19	—	—	Gland weighed 162 gram. and contained 0.13 mg./gram.
H. P.	7	F.	26	+27	—	—	—	—	Small nodule 0.11 mg./gram.; large nodule 0.10 mg./gram.
B. E.	8	F.	39	+ 8	115	28	—	—	—
C. L.	9	F.	25	+10	—	35	29	64	—
S. C.	10	F.	20	+ 0	—	36	32	68	—
R. M.	11	F.	35	+ 5	107	79	28	107	—
G. P.	12	F.	20	+20	176	9	2	11	Gland weighed 225 gram.: iodine content 0.11 mg./gram.
M. M.	13	F.	43	+14	100	65	—	—	Gland weighed 69 gram.: a large gelatinous nodule contained 0.16 mg./gram.: a red granular nodule 0.11 mg./gram.
T. M.	14	F.	30	+14	180	20	18	38	—
M. C.	15	F.	38	+ 0	53	20	13	33	—
H. M.	16	F.	23	+10	411	53	—	—	—
D. M.	17	F.	26	+30	106	—	—	—	—
R. M.	18	F.	25	+17	140	—	—	—	Normal tissue 0.40 mg./gram.: nodule 0.13 mg./gram.
C. E.	19	F.	38	+ 5	24	11	1	12	—
M. M.	20	F.	26	+20	143	—	—	—	—
C. E.	21	F.	21	- 7	374	26	—	—	Nodule 0.42 mg./gram.: adjacent tissue 0.152 mg./gram.
T. R.	22	F.	29	+ 7	23	—	—	—	—
S. M.	23	F.	34	+ 6	33	—	—	—	Gland contained 0.15 mg./gram.
C. H.	24	F.	35	+24	101	—	—	24	Gland weighed 76 gram.: iodine content 0.355 mg./gram.
S. S.	25	F.	20	+ 2	145	—	—	—	—
P. M.	26	F.	49	+12	128	34	11	45	—
J. J.	27	F.	42	+12	95	—	—	—	Nodule contained 0.24 mg./gram.: left lobe 0.32 mg./gram.; right lobe 0.23 mg./gram.
R. E.	28	F.	62	+27	—	—	—	—	Normal tissue 1.28 mg./gram.: nodule 0.24 mg./gram.
L. A.	29	F.	29	+14	—	98	22	120	Gland contained 0.14 mg./gram.
B. T.	30	M.	62	+22	118	84	4	88	Normal tissue 2.18 mg./gram.: nodule 2.12 mg./gram. (after treatment with Lugol)
M. A.*	31	F.	36	+20	328	199	43	242	Iodised salt
T. A.	32	M.	25	+ 7	134	38	—	—	Gland contained 0.42 mg./gram.
P. C.	33	M.	40	+ 5	158	35	9	44	Gland contained 0.53 mg./gram.

\* Indicates iodine treatment.



Hospital. The data were collected and the classification adopted in connection with the Goitre Clinic, Otago Medical School, whose complete findings will be published elsewhere. In the case of those patients who underwent operation, the iodine content of the thyroid gland is given. The basal metabolic rate of all patients is also recorded. Complete analytical data could not be obtained in all cases, on account of difficulties in obtaining specimens for analysis. As regards iodine intake apart from treatment, the patients, while in hospital, received a well-balanced diet which would provide on an average about 50 microgm. of iodine per day.

Table XIII A gives the data for a group of simple colloid goitre. The average age is 25 years, the average basal metabolic rate +9; if cases receiving iodine, together with the anomalous value for patient no. 2 are excluded, the average value for blood iodine is 81 microgm. per kg., and the average daily excretion is 39 microgm. Non-goitrous human blood normally contains about 130 microgm. per kg. The iodine content of the glands in cases 1 and 2 is seen to be very low; according to Marine (1927) a normal gland should contain at least 1 mg. of iodine per grm. of dried gland.

In Table XIII B showing simple nodular goitre which is prevalent in New Zealand, the average age is 34 years and the average basal metabolic rate +12. Blood iodines above 200 microgm. per kg. are rare and obviously exceptional. If these are excluded the average blood iodine is then about 110 microgm. per kg. Similarly the average value of daily excretions below 100 microgm. is 44 microgm. The iodine content of the gland is distinctly

Table XIII c. *Primary Graves' disease.*

Patient	No.	Sex	Age	Basal metabolic rate (mean value)	Iodine content of blood (micro-grm. per kg.)	Iodine excreted in urine (micro-grm. per 24 hrs.)	Iodine excreted in faeces (micro-grm. per 24 hrs.)	Total iodine excreted per 24 hrs. (micro-grm.)	Observations on iodine treatment, if any, and iodine content of gland in cases of operation (dried gland)
McA. G.*	1	M.	28	+60	150	322	121	443	Received Lugol's solution 10 minims T.D.S. Gland weighed 145 grm.: right lobe contained 1.03 mg./grm.; left lobe 1.14 mg./grm.
McI.*	2	F.	21	+42	130	163	50	213	Received Lugol's solution: gland contained 0.717 mg./grm.
C. R.	3	M.	40	+42	136	188	94	282	— —
E. M.	4	F.	49	+69	135	270	120	390	— —
H. S.	5	F.	48	+39	—	27	9	36	— —
D. C.*	6	F.	48	+52	876	3384	107	3491	Received Lugol's solution: gland contained 1.81 mg./grm.
R. A.*	7	F.	42	+38	333	54	77	131	Received Lugol's solution
M. B.	8	F.	15	+43	73	18	24	42	Gland contained 0.37 mg./grm.: adenoma 0.13 mg./grm.
R. M.	9	F.	41	+47	—	—	—	—	Gland weighed 43 grm.: normal tissue 0.49 mg./grm.: nodule 0.47 mg./grm.
S. J.	10	F.	32	+55	30	20	13	33	— —
S. S.*	11	F.	55	+74	677	4941	64	5005	Received Lugol's solution
B. L.	12	F.	57	+96	54	—	—	—	— —

\* Indicates iodine treatment.

Table XIII D. *Secondary Graves' disease ("toxic adenoma")*.

Patient	No.	Sex	Age	Basal metabolic rate (mean value)	Iodine content of blood (micro-grm. per kg.)	Iodine excreted in urine (micro-grm. per 24 hrs.)	Iodine excreted in faeces (micro-grm. per 24 hrs.)	Total iodine excreted per 24 hrs. (micro-grm.)	Observations on iodine treatment, if any, and iodine content of gland in cases of operation (dried gland)
N. A.	1	F.	32	+20	118	—	—	—	—
H. R.	2	F.	39	+35	181	—	—	—	Gland weighed 165 grm.: normal tissue 0.27 mg./grm.: nodule 0.02 mg./grm.
G. E.	3	F.	37	+53	29	—	—	—	—
F. J.	4	F.	32	+46	246	36	—	—	Gland weighed 101 grm.: colloid (granular) 0.54 mg./grm.: nodule (degenerated) 0.25 mg./grm.
W. E.	5	F.	36	+69	102	—	—	—	—
P. E.	6	F.	43	+41	31	23	31	54	—
M. M.	7	F.	30	+28	68	—	—	23	Gland contained 3.26 mg./grm. (after pre-operative Lugol administration)
F. A.	8	F.	51	+37	56	—	—	—	—
S. L.	9	F.	23	+77	237	—	—	—	—
D. V.	10	F.	45	+26	105	912	49	961	—
M. I.	11	F.	52	+42	174	26	8	34	Gland contained 0.82 mg./grm. and nodule 0.56 mg./grm. (after pre-operative Lugol administration)
T. K.*	12	F.	36	+53	82	423	37	460	Receiving iodine injection: gland contained 0.87 mg./grm. and nodule 1.23 mg./grm.

\* Indicates iodine treatment.

low, most frequently lying between 0.11 and 0.20 mg./grm. The nodules, as a general rule, contain a lower concentration of iodine than the normal tissue.

Tables XIII c, d, give observations on cases of Primary and Secondary Graves' disease. The average age for the primary cases is 40 years and the average basal metabolic rate is +55; for the secondary 38 years and +44. The natural iodine content of the blood in cases shown in Table XIII c is too frequently obscured by the effects of iodine treatment for its value to be estimated. In Table XIII d the average blood iodine is not far from normal, but it will be seen that there are several cases in which the blood iodine is abnormally high. This accords with the observations of Veil and Sturm (1925) that the blood iodine is raised in Graves' disease. On the other hand, it is noteworthy that in both tables there are cases in which the blood iodine is distinctly low. Such cases seem to support the theory of a "dys-thyroidism" or qualitative change in the thyroid secretion advanced by Williamson, Plummer, De Quervain and others (1927). The analyses of glands recorded in Tables XIII c, d, show that the untreated gland is always low in iodine, but in cases which have received iodine the iodine content is generally raised above normal. It has been shown that the thyroid gland in Graves' disease, though low in iodine, is extremely active in producing metamorphosis in tadpoles, and this is supposed to be further evidence for the existence of a dys-function (Müller, 1927).

## VI. MISCELLANEOUS.

(1) *Distribution of iodine in the organs and tissues of the white rat.*

An investigation of the distribution of iodine in the different organs and tissues of the white rat was carried out by one of us (H. M. S. T.). One object of the experiment was to determine the normal ratio of the thyroid iodine to the total bodily iodine of the rat. Fellenberg (1926), from a detailed examination of the iodine distribution in guinea-pigs on normal and iodine-rich diets, concluded that the thyroid iodine ordinarily constitutes less than 1 per cent. of the total bodily iodine. The bulk of additional diet iodine was found to be distributed in the skin, hair, flesh and lungs of the animals, though high concentrations were found in the organs, particularly the spleen.

Studies of the thyroids and bloods of various animals have led us to the conclusion that the guinea-pig is an exceptional animal in this respect when compared with rats, hares, rabbits, etc.; and, indeed, in a much later publication Fellenberg (1930) states that thyroid iodine may normally constitute 5-20 per cent. of the total bodily iodine for certain animals.

The present experiments were carried out on three litters of rats, *A*, *B* and *C*. All three groups were fed on white bread and milk for 8 weeks (Period I), their daily iodine intake being 0.33 microgram. per rat. During the next period of 5 weeks (Period II) *B* and *C* had a daily addition to their diet of 1 microgram. of iodine in the form of potassium iodide and thyroid extract respectively, which raised their intake to 1.33 microgram. per rat per day. The ordinary diet was then resumed in all groups for 8 weeks (Period III), and this was followed by another 5-week period with an additional microgram. of iodine, but in this case *B* received thyroid extract and *C* potassium iodide (Period IV). Finally, there followed a 7-week period on the ordinary diet (Period V). Group *A* was kept on ordinary diet throughout.

At the end of the second and succeeding periods a rat from each group was killed and a partition analysis carried out. The results are shown in Tables XIV A, B, C.

Table XIV A contains the data for rats at different ages on a diet rather low in iodine. It is seen that thyroid iodine for these rats averages 10 per cent. of the total bodily iodine, which has an average value of 10 microgram. Examination of Table XIV B shows that, in rats which received additional iodine, the thyroid iodine represents on an average 17 per cent. of the total bodily iodine which itself averages 23 microgram. Administration of iodide leads to slightly greater storage as compared with thyroid extract; otherwise the two substances are similar in their effects. Finally Table XIV C shows that, for rats on a low iodine diet, following a diet higher in iodine, thyroid iodine averages 12 per cent. of the total bodily iodine, which has an average value of 17 microgram. These last results may be taken as normal values of thyroid percentage and total iodine for the adult white rat.

Table XIV A. *Group A receiving 0.33 microgm. per rat per day.*

Period ...	End of II (age 19 weeks)			End of III (age 27 weeks)			End of IV (age 32 weeks)			End of V (age 39 weeks)		
Sex ... ..	F.			F.			F.			M.		
Weight (gram.)	133			144			148			213		
Iodine in organs.												
Organ	I	II	III	I	II	III	I	II	III	I	II	III
	Total iodine in organ (micro-gram.)	% of bodily iodine	Conc. in micro-gram. per gram.	Total iodine in organ (micro-gram.)	% of bodily iodine	Conc. in micro-gram. per gram.	Total iodine in organ (micro-gram.)	% of bodily iodine	Conc. in micro-gram. per gram.	Total iodine in organ (micro-gram.)	% of bodily iodine	Conc. in micro-gram. per gram.
Thyroid	1.2	13	80	1.6	14	90	0.6	5	38	0.8	9	30
Skin	2.7	28	0.1	5.2	45	0.2	2.2	20	0.1	2.1	24	0.1
Carcass	2.2	28	<0.1	2.4	21	<0.1	2.6	24	<0.1	1.9	21	<0.1
Liver	1.3	1.4	0.2	0	0	0	0	0	0	1.3	14	0.1
Spleen	0.4	5	1.0	0	0	0	0	0	0	0	0	0
Genitals	0	0	0	0.7	6	0.2	2.6	24	0.6	1.7	19	0.2
Heart and lungs	1.3	14	0.5	0.8	7	0.4	0.9	8	0.2	0.5	6	0.1
Kidneys and suprarenals	0	0	0	0	0	0	1.3	12	0.8	0	0	0
Alimentary tract	0.4	4	<0.1	0.8	7	0.1	0.7	6	<0.1	0.7	8	<0.1
Total	9.5	—	—	11.5	—	—	10.9	—	—	9.0	—	—

Table XIV B. *Rats receiving 1 microgm. additional iodine as potassium iodide or thyroid extract (1.33 microgm. per rat per day).*

Group ...	B (KI)			C (thyroid extract)			B (thyroid extract)			C (KI)		
Period ...	End of II (age 19 weeks)			End of II (age 19 weeks)			End of IV (age 32 weeks)			End of IV (age 32 weeks)		
Sex ...	F.			F.			F.			F.		
Weight (grm.)	125			144			144			160		
Iodine in organs.												
Organ	I	II	III	I	II	III	I	II	III	I	II	III
	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.
Thyroid	4.3	20	350	3.6	22	250	3.3	12	270	3.5	13	320
Skin	1.8	9	0.1	2.9	18	0.1	7.1	25	0.3	3.6	14	0.1
Carcass	7.7	36	0.1	4.2	25	0.1	4.8	17	0.1	2.5	10	<0.1
Liver	1.8	9	0.2	0.8	5	0.2	1.8	6	0.3	1.0	4	0.1
Spleen	1.9	10	8.0	0.8	5	3.6	2.0	7	8.0	1.6	6	4.2
Genitals	0.7	<1	0.3	1.3	8	0.6	3.2	11	1.0	5.1	20	1.1
Heart and lungs	1.8	9	1.0	1.7	10	0.6	2.2	8	0.6	2.2	8	0.5
Kidneys and suprarenals	0.6	3	0.4	0.5	3	0.4	3.3	12	2.3	4.8	18	3.0
Alimentary tract	0.9	4	0.1	0.7	4	<0.1	1.0	4	0.1	1.7	7	0.1
Total	20.5	—	—	16.5	—	—	28.7	—	—	26.0	—	—

Examination of the other details of distribution in Tables XIV A, B shows that the largest proportion of the total iodine in the case of six rats out of twelve was located in the skin and hair, in four in the carcass, and in two in the genital organs. The highest concentration of iodine apart from the thyroid is found in the spleen.

In earlier experiments with rats under conditions of iodine intake further removed from the normal than those just described we obtained somewhat

Table XIV c. *Rats receiving 0.33 microgm. per rat per day, subsequent to a higher iodine diet of 1.33 microgm. per rat per day.*

Group ...	B			C			B			C		
Period ...	End of III (age 27 weeks)			End of III (age 27 weeks)			End of V (age 39 weeks)			End of V (age 39 weeks)		
Sex ...	F.			F.			M.			M.		
Weight (grm.)	133			140			226			226		
Iodine in organs.												
Organ	I	II	III	I	II	III	I	II	III	I	II	III
	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.	Total iodine in organ (micro-grm.)	% of bodily iodine	Conc. in micro-grm. per grm.
Thyroid	3.0	18	170	2.4	14	150	1.6	10	70	1.2	7	50
Skin	6.1	36	0.2	6.1	36	0.2	2.0	12	0.1	2.8	16	0.1
Carcass	3.0	18	<0.1	3.1	18	<0.1	2.4	15	<0.1	4.0	22	<0.1
Liver	0	0	0	1.7	10	0.3	1.4	8	0.1	2.1	12	0.2
Spleen	0.4	3	1.1	0.4	2	1.3	2.5	15	6.0	1.2	7	2.4
Genitals	1.4	8	0.4	1.3	8	0.4	2.8	17	0.4	2.4	14	0.3
Heart and lungs	0.9	5	0.4	0.9	5	0.4	0.7	4	0.2	0.8	5	0.1
Kidneys and suprarenals	0.5	3	0.3	0.5	3	0.3	2.0	12	0.7	2.0	11	0.8
Alimentary tract	1.7	10	0.2	0.8	5	0.1	1.2	7	<0.1	1.2	7	<0.1
Total	17.0	—	—	17.2	—	—	16.6	—	—	17.7	—	—

different results. Thus with a group of immature rats on a low iodine diet (determined as 0.2 microgm. per rat per day) the values shown in Tables XV A, B, were obtained.

Table XV A. *Rats on low iodine diet (0.2 microgm. per rat per day).*

Rat No. ...	1		2		3		4	
	micro-grm.	micro-grm.	micro-grm.	micro-grm.	micro-grm.	micro-grm.	micro-grm.	micro-grm.
Thyroid wt.	0.011	—	0.014	—	0.0089	—	0.014	—
Iodine content (micro-grm./gm.)	—	170	—	83	—	180	—	99
Iodine in thyroid	—	1.87	—	1.2	—	1.60	—	1.38
Body weight	101	—	123	—	92	—	86	—
Iodine in body	—	1.33	—	2.1	—	3.15	—	1.36
Total iodine in gland and body	—	3.20	—	3.3	—	4.75	—	2.74
Thyroid iodine as percentage of total iodine	58		37		34		50	

Table XV B. *Details of distribution for rat 4 in Table XV A.*

Tissue	Weight in gm.	Total iodine in microgm.	% of total bodily iodine	Conc. of iodine (microgm./gm.)
Thyroid	0.014	1.38	50	99
Skin	15	0.25	9	0.017
Carcass	56	0.40	15	0.007
Liver	6	0.24	9	0.04
Alimentary tract	8.5	0.24	9	0.021
Spleen	0.5	0.23	8	0.46
Total	86	2.74	—	—

Here the thyroid iodine averages 45 per cent. of the total bodily iodine which itself has the very low average figure of 3.5 microgram.

A group of rats on a high iodine diet (about 5 microgram. per rat per day) yielded the results shown in Tables XV.c, D, where the thyroid iodine averages 27 per cent. of the total iodine, and the total iodine averages 28 microgram.

Table XV c. *Rats on high iodine diet (5 microgram. per day).*

Rat No. ...	1		2		3		4	
	gram.	micro-gram.	gram.	micro-gram.	gram.	micro-gram.	gram.	micro-gram.
Thyroid weight	0.01	—	0.0078	—	0.0065	—	0.0072	—
Iodine content (micro-gram./gram.)	—	470	—	1070	—	1140	—	1400
Iodine in thyroid	—	4.7	—	8.35	—	7.4	—	10.1
Body weight	120	—	152	—	139	—	143	—
Iodine in body	—	16.0	—	17.1	—	27.9	—	20.0
Total iodine in gland and body	—	20.7	—	25.5	—	35.3	—	30.1
Thyroid iodine as percentage of total iodine	22		33		21		33	

Table XV D. *Details of distribution for Rat 3 in Table XV C.*

Tissue	Weight in gram.	Total iodine in microgram.	% of total bodily iodine	Conc. of iodine (microgram./gram.)
Thyroid	0.0065	7.4	21	1140
Skin	28	18.0	51	0.65
Carcass	89	6.7	19	0.075
Liver	8	0.3	0.8	0.037
Alimentary tract	13.5	2.7	7.6	0.20
Spleen	0.5	0.2	0.6	0.40
Total	139	35.3	—	—

Finally, with a group of rats on a very high iodine intake (100 microgram. or more per rat per day) the values given in Table XV E were obtained. Here the thyroid iodine averaged 1 per cent. of the total iodine which itself averaged 2060 microgram.

Table XV E. *Rats on diet very high in iodine (100 microgram. or more per rat per day).*

Rat No. ...	1		2		3	
	gram.	micro-gram.	gram.	micro-gram.	gram.	micro-gram.
Thyroid weight	0.012	—	0.0085	—	0.013	—
Iodine content (micro-gram./gram.)	—	1240	—	2250	—	1600
Iodine in thyroid	—	14.9	—	19.1	—	20.8
Body weight	137	—	153	—	162	—
Iodine in body	—	1500	—	2110	—	2520
Total iodine in gland and body	—	1515	—	2129	—	2541
Thyroid iodine as percentage of total iodine	1		0.9		0.8	

Hence, apart from the apparently anomalous results observed in the case of the immature rats, we may conclude that in general the percentage of thyroid iodine varies with the value for total iodine in the rat. The percentage has a minimal value where the total bodily iodine is in the neighbourhood of 10 microgram., it increases with the total iodine to a normal value of about 12 per cent. where the total iodine is about 17 microgram., it rises to a maximum when the total iodine is in the neighbourhood of 30 microgram., and then falls off with greatly increased values of total iodine to about 1 per cent., where it remains approximately constant.

(2) *Iodine content of animal thyroids.*

The iodine content of the thyroid glands of various animals is recorded in Table XVI. The glands were dissected out soon after death and weighed immediately. The iodine is expressed in mg. per gram. of fresh substance.

Table XVI.

Animal	Weight of gland in gram.	Iodine content in mg. per gram.	Weight of animal, with remarks
Monkey ( <i>Macacus rhesus</i> )	0.49 (mean of 10 monkeys)	0.55 (mean of 10)	Average weight of monkeys 3175 gram.
Dog	3.6 (mean of 2 dogs)	0.37 (mean of 2)	Average weight of dogs 4082 gram.
Dog (A)	4.86	0.003	Exhausted condition at death from hydatid toxæmia. Weight of dog 4082 gram.
Lamb	0.85	0.96	Weight 6804 gram. Normal lamb
Lamb (B)	354	0.001	Weight 5443 gram. Large congested goitre
Rabbit	0.095	0.25	Weight 1100 gram. Wild brown rabbit
Rabbit (C)	0.27	0.02	Weight 1200 gram. Laboratory white rabbit
Hare	0.22	0.63	Weight 1500 gram.
Hare (D)	0.18	0.11	Weight 1450 gram.
Rats	0.0140 (mean of 6 rats)	0.136 (mean of 6)	Average weight 200 gram. Low iodine intake
Rats (E)	0.0087 (mean of 6 rats)	0.99 (mean of 6)	Average weight 180 gram. High iodine intake

The weight of the thyroid gland of the dog relative to the weight of the body is worthy of note, it being much greater in the dog than in the monkey. In the case of dog (A) which was in an extreme condition of exhaustion at death from hydatid toxæmia, the gland was of large size and its iodine store was practically depleted. The lamb (B) had a huge congenital goitre which weighed over 350 times more than a normal lamb's thyroid. The thyroid constituted approximately one-fifteenth of the total weight of the animal. It was one of a series of lambs with congenital goitre (forty) born in Otautau in the endemic area of Southland. There is a striking difference in the weight and iodine content of a wild rabbit and a laboratory reared rabbit. The hare thyroids were obtained at a hare drive in Otago at mid-winter. It will be seen that hare (D) contained less than one-fifth the amount of iodine per unit

weight of gland as compared with the other, and histologically it presented the typical appearance of the secretory phase of Williamson and Pearce (1928). It is possible that one animal may have been shot early in the drive and the other towards the end, and that the factors of fear and exhaustion may have been responsible for the striking difference in histology and iodine content. This subject is receiving further consideration. It will be seen that the rats on low iodine diet (bread and milk) show thyroids of nearly twice the weight and of approximately one-seventh the iodine content.

(3) *Variations in the iodine content of iodised salts.*

Previous studies on the iodine content of domestic salt as used in New Zealand have shown that it has an extremely low iodine content. Several samples contained less than one microgm. per kg. One sample of Fijian marine salt contained 20 microgm. per kg., but it was not in general domestic use. Crude Rock Salt was also found to have a low content varying from 0 to 10 microgm. per kg. The only samples which were uniformly high were obtained from naturally occurring salt licks which were eagerly sought after by sheep and cattle in the endemic areas of Otago and Marlborough. These salts contained as much as 9000 microgm. per kg. The introduction of artificially iodised salts into the country standardised officially to contain one part of potassium iodide per 250,000 parts prompted us to carry out a series of tests on various samples of iodised salts to determine to what extent it is possible for the manufacturers to conform to the standard. In addition to the extraction method of analysis titration was carried out by the usual method, viz. the potassium iodide was oxidised to iodate in acid solution with bromine and the iodine titrated with thiosulphate solution after its liberation by the addition of iodide and phosphoric acid. The results are shown in Table XVII.

Table XVII. *Iodine content of iodised salts.*

Specimen	Parts of KI per 250,000 parts of salt	
	Extraction method	Titration method
Union iodised salt (average of 8 samples)	0.6	0.7
Imperial brand (average of 2 samples)	0.9	1.1
Cerebos iodised salt (average of 6 samples)	0.5	0.7
Windsor iodised salt (average of 8 samples)	0.5	0.6
Salodine iodised salt (average of 2 samples)	0.8	0.8

It will be seen that practically all the samples are fluctuating below the standard. During the course of the analyses two samples of different salts were found to contain 2.5 and 6.9 parts respectively per 250,000, thus indicating the need for careful analytical control.

An investigation was therefore undertaken to determine to what extent the potassium iodide was distributed uniformly throughout samples of salt



supplied in bags. Samples of iodised salt containing 1.23 parts of KI per 250,000 stored in corked bottles in the laboratory had previously been found to have the same iodine content at the end of 12 months' storage. Samples of salt were obtained from various positions in various sized bags and the findings are shown in Table XVIII.

Table XVIII. *Distribution of iodine in various portions of bulk samples of iodised salt.*

No.	Specimen	Wt. of bag (lb.)	Region of bag	Parts KI per 250,000 salt
1	Windsor iodised salt	5	Top	0.47
			Bottom	0.24
2	Windsor iodised salt	5	Top	0.46
			Bottom	0.49
3	Windsor iodised bakers' salt	112	Top	0.51
			Side halfway down	0.53
			Side near bottom	0.96
			Bottom corner	0.40

It will be seen from Table XVIII that in one 5 lb. bag the top sample contained twice as much iodine as the sample from the bottom, whereas in another similar bag the samples from top and bottom were practically identical. A large 112 lb. bag of bakers' iodised salt showed significant variation; a sample from the bottom corner of the bag having half the iodine content of an adjacent sample situated 4 inches further in and above. This finding suggests that the variation may be due to some factor other than the uneven distribution of the potassium iodide during manufacture. V. Fellenberg (1926) has shown that traces of water in conjunction with the extreme solubility of potassium iodide may cause re-distribution of the iodide both by gravitational and capillary processes. Johnson and Herrington (1927) concluded that considerable loss of iodine occurred if free circulation of air was permitted by storing in sacking. The minimum loss occurred when storage was carried out in an atmosphere of relative humidity of 50 per cent. Iodised salt rendered alkaline by the addition of sodium bicarbonate (1 per cent.) loses only insignificant amounts of iodine on prolonged exposure to sunlight and to heat (80° C.). Neutral or slightly acid specimens under these conditions lose relatively large quantities of iodine.

(4) *Amount of iodine returned to the soil by rain water.*

In a previous study (Hercus, Benson and Carter, 1925) the extent to which the soil of coastal districts might obtain iodine from rain and from sea spray was discussed. Regular samples of rain water were obtained from Hokitika, which is situated on a narrow coastal plain of the South Island of New Zealand, at the foot of the Southern Alps facing the rain-bearing winds of the Tasman Sea, and has an annual rainfall of 200 inches, and from Central Otago, which is an inland plateau 50 miles distant from the sea, and with an annual rainfall of 13-20 inches.

The iodine and chloride contents of the various samples were determined and the findings are given in Table XIX.

Table XIX.

Place	Date	Rainfall in inches	Chloride NaCl/10 <sup>4</sup> mg.	Iodine in microgm. per litre
Hokitika	27. vii.-11. ix. 26	—	15.4	—
	12. ix.-15. x. 26	—	18.4	—
	16. x.-1. xi. 26	—	8.4	—
	2-14. xi. 26	8.07	9.6	6.8
	15. xi.-6. xii. 26	7.56	—	—
	7. xii. 26-15. i. 27	8.11	15.8	1.8
	16. i.-2. ii. 27	7.96	—	2.5
	3. ii.-15. iii. 27	8.14	—	3.0
	16. iii.-27. iii. 27	8.54	—	1.8
	28. iii.-23. iv. 27	8.44	14.4	3.4
	24. iv.-19. v. 27	7.49	13.9	3.1
	20-28. v. 27	8.29	10.3	0.9
	29. v.-25. vi. 27	4.14	9.1	1.6
	3. x.-4. xi. 26	8.38	1.18	—
	4. xi. 26-1. ii. 27	8.05	11.5	4.8
	1. ii.-24. iv. 27	8.70	25.0	2.1
Weraroa (Central Development Farm)	24. iv.-9. vi. 27	8.86	6.2	2.4
	23. vi.-5. xii. 26	6.94	1.4	1.2
Ophir	7. xii. 26-25. iii. 27	8.20	—	—
	26. iii.-7. xii. 27	7.76	1.0	—

From Table XIX it will be seen that the iodine content of all the samples is appreciable and in some samples relatively high. The average ratio of iodine to chlorine is about 1 to 2500 which is of a distinctly higher order than the ratios determined by McClendon and Hathaway (1924) and Fellenberg (1924).

There is, however, no indication of even approximate constancy in the iodine-chlorine ratio.

## VII. SUMMARY.

1. Most artificial manures are found to have some effect in increasing the iodine content of crops, particularly superphosphate.

2. Marked differences are found between the iodine contents of Samoan and New Zealand foodstuffs corresponding to the non-goitrous condition of Samoa and the partial endemicity of New Zealand.

3. Investigation of the dietaries of fifteen residential institutions showed that improvement in provision of iodine was necessary in many cases by using fish at least twice a week, raising the daily ration of milk to 1 pint per head, and employing iodised salt for table and culinary purposes.

4. Dietetic experiments indicate (i) that on a change of diet adjustment of iodine metabolism may take a considerable time, (ii) that iodised salt is most effective in promoting storage of iodine when associated with a meat diet.

5. Iodine excretion is found to decrease much more rapidly under fasting than on a diet very low in iodine.

6. Iodised salt has the effect of adjusting the excretion of goitrous adults to the normal non-goitrous value.

7. The iodine content of the blood is found to be lower than normal in cases of simple colloid and simple nodular goitre, though it may range from low to abnormally high in cases of Graves' disease. In all types of goitre the thyroid gland is low in iodine.

8. The ratio of thyroid iodine to total bodily iodine is about 12 per cent. in the case of the white rat under ordinary conditions, but the ratio is found to vary considerably with the iodine intake.

9. Great differences in the ratio of the weight of the thyroid gland to that of the whole body are found among different species of animals, the ratio being higher for the dog than for the monkey.

10. Variations in commercial samples of iodised salts are recorded and discussed.

11. Data are given in regard to the iodine and chlorine content of rain-water in different localities.

#### REFERENCES.

- DE QUERVAIN (1927). *C.R. Conf. Int. Goitre*, Berne, p. 134.  
FELLENBERG (1923-4). Studies in *Biochem. Zeitschr.* **139**, 142, 152.  
—— (1926). *Ergbn. d. Physiol.* **25**, 176.  
—— (1930). *Biochem. Zeitschr.* **217**, 227.  
HERCUS, BENSON and CARTER (1925). *J. Hygiene*, **24**, 321-402.  
HERCUS and ROBERTS (1927). *Ibid.* **26**, 49.  
JOHNSON and HERRINGTON (1927). *J. Agric. Research*, **35**, 167.  
LEITCH and HENDERSON (1926). *Biochem. J.* **20**, 1003.  
MARINE (1927). *C.R. Conf. Int. Goitre*, Berne, p. 68.  
MCCLENDON and HATHAWAY (1923). *Proc. Soc. Exper. Biol. and Med.* **21**, 129.  
—— (1924). *J. Amer. Med. Ass.* **82**, 1668.  
MEYER and KIRSCHBAUM (1928). *Zeitschr. Anal. Chem.* **73**, 321.  
MÜLLER (1927). *C.R. Conf. Int. Goitre*, Berne, p. 221.  
ORR, KELLY and STUART (1928). *J. Agric. Soc.* **18**, 159.  
ORR and LEITCH (1929). Iodine in Nutrition. *Medical Research Council Report*, No. 123.  
SCHARRER and STROBEL (1927). *Angew. Botanik*, **9**, 187.  
STORMS and TODHUNTER (1928). *J. Home Economics*, **20**, 817.  
VEIL and STURM (1925). *Dtsch. Arch. f. klin. Med.* **147**, 166.  
WILLIAMSON and PEARCE (1928). *J. Pathol. and Bacteriol.* **31**, 255.

## APPENDIX.

*Diet A.*

Food	Weight in grm.	Calories	Protein in grm.	CaO in grm.	P <sub>2</sub> O <sub>5</sub> in grm.	Fe in grm.	Iodine in microgrm. per kg.	Iodine in microgrm.
Milk	80 c.c.	55	2.70	0.134	0.169	0.00019	15	1.20
Meat	60	200	11.40	0.009	0.276	0.00170	9.5	0.57
Butter	54	415	0.54	0.012	0.021	0.00012	5	0.27
White bread	180	474	16.60	0.071	0.384	0.00166	2	0.36
Sugar	25	100	0	0	0	0	0	0
Rice	18	62	1.44	0.002	0.039	0.00016	2	0.04
Kornies	10	37	1.05	0.006	0.075	0.00046	20	0.20
Biscuit	12	50	1.80	0.004	0.028	0.00018	2	0.02
Potatoes	90	75	1.98	0.021	0.142	0.00140	6	0.54
Cabbage	130	41	2.08	0.080	0.084	0.00139	20	2.60
Carrots	42	19	0.46	0.035	0.046	0.00027	6	0.25
Apples	108	68	0.43	0.011	0.031	0.00033	3	0.32
Pears	83	53	0.50	0.018	0.049	0.00025	3	0.25
Peaches	75	31	0.53	0.016	0.040	0.00023	3	0.23
Banana	73	72	0.95	0.009	0.052	0.00044	3	0.22
Jam	30	100	0.20	0.028	0.089	0.00069	6	0.18
Marmalade	30	100	0.16	0.082	0.061	0.00026	6	0.18
Water	675 c.c.	—	—	—	—	—	1	0.68
Total	—	1952	42.82	0.538	1.596	0.00973	—	8.11

*Diet B.*

Food	Weight in grm.	Calories	Protein in grm.	CaO in grm.	P <sub>2</sub> O <sub>5</sub> in grm.	Fe in grm.	Iodine in microgrm. per kg.	Iodine in microgrm.
Milk	475 c.c.	327	16.10	0.795	1.007	0.00114	15	7.13
Meat	60	200	11.40	0.009	0.276	0.00170	9.5	0.57
Tinned fish	55	112	12.10	0.017	0.301	0.00065	500	27.50
Butter	48	369	0.48	0.001	0.002	0.00001	5	0.24
Custard	36	50	4.80	0.031	0.139	0.00102	30	1.08
Brown bread	180	530	16.00	0.148	1.010	0.00508	10	1.80
Sugar	25	100	0	0	0	0	0	0
Kornies	10	37	1.05	0.006	0.075	0.00046	20	0.20
Potatoes	100	83	2.20	0.023	0.158	0.00150	6	0.60
Cabbage	155	49	2.48	0.098	0.103	0.00171	20	3.10
Lettuce	40	8	0.48	0.026	0.043	0.00066	12	0.48
Pears	90	57	0.54	0.018	0.057	0.00026	3	0.27
Dates	36	124	0.76	0.032	0.046	0.00107	12	0.43
Figs	65	200	2.79	0.047	0.054	0.00062	12	0.78
Banana	63	62	0.82	0.007	0.045	0.00038	3	0.19
Marmalade	30	100	0.16	0.082	0.061	0.00026	6	0.18
Water	500 c.c.	—	—	—	—	—	1	0.50
Total	—	2408	72.16	1.340	3.377	0.01652	—	45.05

*Diet C.*

Food	Weight in grm.	Calories	Protein in grm.	CaO in grm.	P <sub>2</sub> O <sub>5</sub> in grm.	Fe in grm.	Iodine in microgrm. per kg.	Iodine in microgrm.
Milk	125 c.c.	86	4.24	0.209	0.285	0.00030	15	1.89
Meat	150	500	28.50	0.022	0.890	0.00425	8	1.20
Butter	55	423	0.55	0.012	0.021	0.00012	12	0.66
White bread	153	403	14.11	0.061	0.328	0.00141	2	0.31
Sugar	25	100	0	0	0	0	0	0
Sago	13	45	0.05	0.003	0.026	0.00020	2	0.03
Porridge	25	100	4.20	0.024	0.226	0.00096	30	0.75
Potatoes*	200	186	4.40	0.046	0.316	0.00300	9	1.80
Cabbage*	100	33	1.60	0.066	0.070	0.00107	7.5	0.75
Apples	60	43	0.27	0.0068	0.019	0.00021	4	0.24
Jam	45	150	0.30	0.042	0.133	0.00103	10	0.45
Water	500 c.c.	—	—	—	—	—	0	0
Iodised salt	7.5	—	—	—	—	—	—	16
Totals	—	2049	58.22	0.4918	2.092	0.01255	—	24.08

\* Water discarded; I<sub>2</sub> lost from salt and from vegetables.

*Diet D.*

Food	Weight in grm.	Calories	Iodine in microgrm. per kg.	Iodine in microgrm.
Milk	306 c.c.	210	15	4.59
Fish	25	—	500	12.5
Butter	52	400	5	0.260
Cheese	5	20	30	0.15
Eggs	30	44	56	1.68
White bread	79	208	2	0.158
Brown bread	95	250	10	0.950
Sugar	36	144	0	0
Rice	30	133	2	0.300
Kornies	20	74	20	0.400
White flour	53	189	2	0.106
Potatoes	140	117	6	0.280
Spinach	100	24	20	2.000
Lettuce	42	8	12	0.504
Apple	170	107	3	0.510
Pear	135	86	3	0.405
Banana	162	159	3	0.486
Iodised salt	7.5	—	—	20.000
Total	—	2173	—	45.379

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# FURTHER STUDIES IN ANTI-RABIES IMMUNISATION. RABIES VIRUS—EXALTED AND CLASSICAL STRAINS COMPARED.

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## GENERAL INTRODUCTION.

In a previous series of studies published by us (1929) it was established that the anti-rabies immunity produced by carbolised fixed virus approximates in practice to that produced in the survivors of animal groups treated with living virus in normal saline solution. Evidence of efficacy of treatment was further sought in an estimation of antibody content in immune serum and this naturally evoked, *inter alia*, an enquiry into the rabicidal properties possessed by such serum. It was found that fresh fixed virus acts as a greater stimulus to the production of both immunity and rabicidal antibody than killed carbolised virus. The degree of immunity and rabicidal action, however, possessed by the sera of rabbits and rats treated with killed carbolised virus proved sufficiently high to confer protection against the street virus used during the investigations and exhibited in a dosage far exceeding that likely to be encountered in natural infections. From the results it appeared that immunity and rabicidal action are produced concomitantly, are present in greatest degree together and disappear at or about the same time. Moreover

it was inferred, although final proof was wanting, that the rabicidal properties of an animal's serum, are, in general, an indication of the immunity against rabies possessed by that animal. For obvious reasons, however, the same "standardised" strain of street virus was necessarily employed throughout our experimental work—a strain of proved constant strength in respect of virulence and infective power and one capable of producing, with certainty, in a dilution of 1:1000, symptoms in rabbits on the sixteenth day after subdural inoculation with 0.2 c.c. and death 4 days later. But street virus is derived from many sources and from many species of animals able to convey infection; it need not, therefore, be a matter for surprise that animals immunised in the ordinary way against rabies may yet be unable to survive the critical test of later inoculation with newly discovered, uninvestigated, unknown or vicarious strains of street virus. Experience has abundantly shown that in nature there exists a multiplicity of street viruses distinguishable by certain characteristics but more particularly by individual pathogenicity. Thus extreme variations in virulence are exemplified by the oulou-fato virus of West Africa on the one hand and by the Tangier B (Chabrier) strain on the other, the former, apparently innocuous to man, showing on first transfer to rabbits an incubation period of from 15 to 17 days, the latter one of 8 days only. Such difference, however, purely one of degree of virulence, would constitute no considerable problem in anti-rabies immunisation, but the existence of biological variations among various strains of street virus, the suggestion, indeed, of biological individuality, would, if proved, render of little account most modern methods of Pasteurian treatment and envisage the replacement of monovalent antigens with polyvalent and, where feasible, auto-vaccines.

The occurrence in Palestine during 1930 of a street virus of extraordinary virulence, which caused, despite treatment, the death of three bitten persons, afforded us an opportunity of investigating at first hand the basic question of plurality of viruses. To throw light upon the subject it seemed expedient first to institute enquiry into the nature of such an exalted virus and then to compare its infective, immunising and rabicidal antibody-producing powers with those of the ordinary virus in daily use here. The results of our investigations, which included cross-immunity and inoculation experiments, are given below.

## I. EXALTED RABIES VIRUS (VIRUS DE RUE RENFORCÉ).

### 1. OCCURRENCE.

On the same day in two neighbouring villages in Northern Palestine seven persons were bitten by a rabid jackal, and of that number, despite the administration of a full course of anti-rabies vaccine, three developed symptoms of hydrophobia. Anti-rabies procedure in Palestine includes complete decentralisation of treatment, and the vaccine used in the fifteen district

institutes and prepared for issue at the Central Laboratories consists of a 1 per cent. emulsion of carbolised fixed virus, which is exhibited over a period of 14 consecutive days in a daily dosage of 5 c.c. totalling 0.7 gm. nervous substance. Most probably because of their belonging to the ignorant peasant class all seven bitten persons delayed reporting at the nearest District Health Office until 2 days after the time of biting, thereby rendering cauterisation and other forms of local treatment inadequate.

The following is a brief synopsis of the case histories:

*Case 1.* Male; age 25; bitten severely on nose 2. v. 30; treated between 4. v. 30 and 18. v. 30 but absent on third day of prescribed treatment; appearance of symptoms 19. v. 30 and death 2 days later; autopsy performed and brain forwarded for laboratory examination; microscopic findings: Negri bodies numerous—and animal inoculation tests confirmed the clinical diagnosis of hydrophobia. The incubation period of the disease was, therefore, 17 days and its duration 2 days.

*Case 2.* Male; age 30; received two deep face wounds; attendance at treatment centre identical with that of Case 1; onset of symptoms 30. v. 30 with death next day. In this case the incubation period was 29 days.

*Case 3.* Male; age 4; son of Case 2; showed one deep wound on head; attended for treatment as Cases 1 and 2; date of symptoms 8. vi. 30; duration of disease 10 hours; incubation period 37 days.

*Cases 4, 5, 6, 7,* however, bitten with moderate severity by the same jackal are now, 6 months after the completion of treatment, alive and well. Each of the four commenced treatment 2 days after being bitten and failed to attend on the second, third and fourth days of the prescribed course. These cases may thus be summarised:

Case No.	Sex	Age	Location and nature of wounds
4	M.	60	One wound on head (scalp)
5	M.	25	Four wounds on upper arm through naked skin
6	F.	50	Two wounds on upper arm through clothing
7	M.	45	One wound on head (face)

## 2. RECOVERY OF VIRUS.

On 25. v. 30 the M.O.H. Safad submitted for laboratory examination part of a brain taken from a human case of suspected rabies. This, for Palestine, somewhat unusual procedure was followed in the present instance for two reasons: (a) the patient had been bitten less than 3 weeks before his death by a rabid jackal, and (b) although the symptomatology described by the deceased's relatives was suggestive of hydrophobia, the Medical Officer had had no opportunity of observing the clinical course of the disease.

Results obtained at the laboratory following direct subdural inoculation of rabbits with emulsions of the human brain indicated the atypical nature of the rabies strain—hereafter referred to as the Safad Virus—and led to an enquiry being made into its main characteristics.



## 3. CHARACTERISTICS OF THE SAFAD VIRUS.

Reviewing the work of Busson (1928) and of Schweinburg (1928) on rabies virus, McKendrick (1929) observes that viruses were examined by these authors with regard to (1) infectivity (M.L.D. on subdural inoculation), (2) the incubation period which each induced (related to organ-specificity), and (3) the duration of the resulting illness (a measure of specific toxicity). "These three characteristics are fundamental," he continues, "and in comparing strains of either street or fixed virus their study is essential."

The following is an account of the Safad virus considered mainly from these standpoints.

*(a) Infectivity.*

Measurement of infectivity, then, is usually made by an accurate estimation of the minimal lethal dose, or M.L.D., of the substance under test, *i.e.* the smallest quantity which will, without fail, produce symptoms in a susceptible animal and cause its death in a certain time. As regards the M.L.D. of rabies virus, however, most workers in its determination pay insufficient attention, apparently, to one of its definitive criteria, *viz.* that the time interval between inoculation and appearance of symptoms (or death) must be a constant; indeed, in the evaluation of such viruses, they commonly regard the smallest quantity of rabid nervous tissue capable ultimately of producing symptoms as sole index of virulence. But since it is generally held that rabies virus is dependent for its activity on many unknown or unappreciated factors such as its distribution and concentration in the central nervous system, "lag," its rate of multiplication and its specific aggressiveness, the reason for the elimination from most calculations of the time factor is not immediately obvious. True it is that Schweinburg (1928) in contrasting a number of street virus strains by the effect of their subdural inoculation in varying dilution provided a notable exception; in fact he proved the non-relationship of M.L.D. to incubation period by finding that, with incubation times of 9, 9, 16, 30 and 16 days, the corresponding lethal doses were 1/2000th, 1/5000th, 1/8000th, 1/2000th and 1/7000th mg. respectively. It is submitted that in all similar investigations the definition of M.L.D. given by us (1929) should be strictly adhered to: "the highest dilution of a virus which, in rabbits of average weight (1400 grm.), will invariably produce symptoms in the same period of time after subdural infection with 0.2 c.c." Results of our determination of M.L.D. made in accordance with the requirements of this definition are now given in Table I in respect of (1) the fixed virus strain used here in vaccine preparation, (2) an ordinary Palestinian street virus, and (3) the Safad virus on the days of its second and twenty-ninth rabbit transfers.

From the results given in Table I it will be seen that the true M.L.D. of each virus under test proved to be a 1:1000 dilution.

(b) *Incubation time and duration of illness.*

Multiplication rate (= incubation time) of the Safad virus, together with its deleterious action (= specific toxicity), is shown in Table II, which embodies the findings consequent upon the subdural inoculation of rabbits with the virus during its first twenty subpassages.

Table I. *Estimation of the true M.L.D. of rabies virus.*

No. of rabbit	Dilution of virus used	Day of onset of symptoms after subdural inoculation with				Day of death after subdural inoculation with			
		Fixed virus Paris	Street virus Jaffa	Safad virus		Fixed virus Paris	Street virus Jaffa	Safad virus	
				2nd day	29th day			2nd day	29th day
1	1: 1,000	5	16	6	5	7	20	7	6
2	1: 1,000	5	16	6	5	7	20	7	6
3	1: 1,000	5	16	6	5	8	20	7	6
4	1: 1,000	5	16	6	5	7	20	7	6
5	1: 2,500	5	16	6	6	7	20	7	8
6	1: 2,500	6	18	7	6	8	22	8	8
7	1: 2,500	6	16	7	5	9	20	9	7
8	1: 2,500	5	18	6	6	7	23	7	8
9	1: 5,000	6	17	7	6	8	21	9	9
10	1: 5,000	5	16	8	7	7	21	10	10
11	1: 5,000	6	20	7	7	9	24	9	10
12	1: 5,000	6	18	9	6	8	22	11	9
13	1: 10,000	8	19	7	7	10	24	8	11
14	1: 10,000	6	18	10	9	9	22	13	12
15	1: 10,000	7	20	9	8	10	24	11	11
16	1: 10,000	22	18	11	8	24	23	14	11

Table II. *Incubation time and specific toxicity of Safad virus.*

No. of passage	Date of inoculation	Date of appearance of symptoms	Incubation period (in days)	Date of death	Duration of illness (in days)
1	26. v. 30	2. vi. 30	7	3. vi. 30	1
2	12. vi. 30	18. vi. 30	6	19. vi. 30	1
3	19. vi. 30	25. vi. 30	6	26. vi. 30	1
4	26. vi. 30	3. vii. 30	7	4. vii. 30	1
5	4. vii. 30	10. vii. 30	6	11. vii. 30	1
6	11. vii. 30	17. vii. 30	6	19. vii. 30	2
7	19. vii. 30	25. vii. 30	6	26. vii. 30	1
8	29. vii. 30	4. viii. 30	6	5. viii. 30	1
9	5. viii. 30	10. viii. 30	5	12. viii. 30	2
10	12. viii. 30	17. viii. 30	5	19. viii. 30	2
11	19. viii. 30	24. viii. 30	5	26. viii. 30	2
12	26. viii. 30	31. viii. 30	5	2. ix. 30	2
13	2. ix. 30	7. ix. 30	5	9. ix. 30	2
14	9. ix. 30	14. ix. 30	5	16. ix. 30	2
15	16. ix. 30	21. ix. 30	5	23. ix. 30	2
16	23. ix. 30	28. ix. 30	5	30. ix. 30	2
17	30. ix. 30	5. x. 30	5	7. x. 30	2
18	7. x. 30	12. x. 30	5	14. x. 30	2
19	14. x. 30	19. x. 30	5	21. x. 30	2
20	21. x. 30	26. x. 30	5	28. x. 30	2

Table II shows that the Safad virus, originally obtained from the brain of a person dead from a rabid jackal's bite and thereafter introduced subdurally into rabbit series, first caused illness in 7 days and death in 8. As a result of successive passages through rabbits symptoms appeared in 6, 6, 7, 6, 6, 6, 6, 5, 5, 5, 5, 5 days after inoculation and death occurred in 7, 7, 8, 7, 8, 7, 7, 7, 7, 7, 7 days respectively, the duration of illness varying from 1 to 2 days.

*(c) Occurrence of Negri bodies.*

Histological examination of the human brain submitted for diagnosis showed, with Mann's stain, Negri bodies measuring  $2-6\mu$  in diameter to be fairly numerous in the large pyramidal cells of the hippocampus major lying close to the alveus. The brain of the first rabbit, however, inoculated with a portion of human hippocampus, revealed a total absence of Negri bodies from the pyramidal cells of the cerebral cortex in general and of Ammon's horn in particular and also from Purkinje's layer of corpuscles in the cerebellum. The same complete absence of Negri bodies was observed in the brains of rabbits examined after the second, third, fourth, fifth and sixth subpassages.

## 4. CHARACTERISTICS OF VIRUS DE RUE RENFORCÉ.

Generally speaking, while there exists considerable diversity of opinion regarding the nature, if not the properties of rabies virus, the terminology "street virus" and "fixed virus" has a universal significance and the difference between these viruses is ordinarily held to be qualitative rather than quantitative. Nevertheless, although certain differences may readily be explained on quantitative grounds and/or as a result of adaptation, and although the two viruses on close study present the greatest similarity, there are five distinguishing features commonly advanced as characteristic: mode of transmission and propagation; the presence or absence of Negri bodies; the type of lesion produced in the nerve centres; the length of incubation period and the very definite reduction in pathogenicity for man of fixed virus introduced subcutaneously—an attenuation of virulence practically to the point of innocuity. Of these incubation time commonly serves to differentiate fixed virus from the street virus of its origin and as a criterion of virulence and will, therefore, first be considered.

*(a) Incubation time.*

Evidence of there being reinforced or exalted strains of street virus in nature is afforded by a study of available statistics giving the incubation periods of hydrophobia in man. According to Harvey and McKendrick (1930) "the period of incubation varies with the distance to be travelled (by the virus). In man, for implantation on the head it is approximately 27 days, on the arm 32 days and on the leg 64 days, but these figures are subject to wide variations." Thus, in the experience of Bauer (1886), among 510 cases hydrophobia supervened in 8.24 per cent. during the first 19 days following the bite; Alivisatos (1926) has recorded incubation periods of 10 and 13 days and Konradi (1923) periods of 12, 13 and 14 days respectively. It is the opinion of Lubinski and Prausnitz (1926) and of Alivisatos (1926) among others that these cases might well be explained by an increased virulence of rabies virus.

Further evidence of the occurrence of naturally exalted street viruses is

to be adduced from a consideration of their effects on first transmission to rabbits. Thus Babes (1912), in discussing variations in virulence of rabies virus as it occurs in nature, states that rabbits inoculated subdurally with street virus mostly succumb in about 14 days, this interval between infection and death indicating a virus of average virulence, but, he continues, "il existe en outre des virus naturellement exaltés." According to Helman (1888), ordinary incubation time is from 14 to 18 (20) days, to Lubinski and Prausnitz (1926) from 15 to 20 days, while to Harvey and Acton (1923) "this conception of an average interval of 15 days' incubation is taken for granted by practically all institutes."

Now, if from the brain of a rabbit dead from the street virus strains ordinarily encountered transference by the subdural route is effected into another rabbit and if this process is repeated in successive transfers from one rabbit series to another, it will be found that, in the course of such sub-passaging, the incubation period consistently shortens until at about the fiftieth passage it achieves a constant minimum of 6 or 7 days (absolute limits are (3) 4 and 10 days). The average incubation time, therefore, for street virus may be put at 15 days and for fixed virus (6) 7.

But in nature the rare occurrence of street virus strains with, when compared with fixed virus, identical shortness of incubation period has long since been recognised. Thus Remlinger (1908), while examining cases of rabies in very young dogs, more than once obtained a strain which, on first transmission to rabbits, caused death in from 8 to 10 days. Such a strain termed "virus (de rue) renforcé" had previously been encountered and recorded by Calabrese (1896), Abba (1898) and d'Amato (1904), Calabrese having found, for example, that of 280 street viruses investigated at the Pasteur Institute in Paris twenty or 7 per cent. produced symptoms in rabbits within the first 10 days. Since then Remlinger (1924) in Morocco, Alivisatos (1926) in Serbia, Koch (1929) in Germany and Harvey and Acton (1923) in India have added to the list of street viruses belonging to this category. In the case of the Moroccan virus, originally derived from a young fox, rabbits inoculated with the strain developed symptoms on the eighth day and died on the ninth; the Servian strain also, with an incubation period of 8 days following intramuscular injection, exemplifies a street virus of specially high virulence. In Koch's list are included several strains of typical hyper-virulence. Thus, for instance, a street virus obtained from a cat invariably killed experimental animals after an incubation period of 5 days, behaving from the first passage as a fixed virus. Again, from a human case of hydrophobia, in which symptoms had developed 22 days after the bite, Koch recovered a strain, which proved, on rabbit inoculation, stronger than the fixed virus he used for prophylactic vaccination, all animals dying in the second passage after an incubation period of 3 days. Other reinforced viruses gave incubation times varying from 5 to 7 days on primary rabbit transfer. Further, the records of Harvey and Acton (1923) show that 650 street viruses have been investigated at Kasauli with incuba-

tion periods varying from 5 to 61 days, and of that number onset of paralytic symptoms was observed during the first 10 days in ninety-three cases or in 14.3 per cent. Our experience in Palestine, however, in contrast to the findings of Harvey and Acton, tends to support the views expressed by Remlinger (1908, 1924) and Babes (1912) that occurrence of exalted virus is somewhat infrequent. Thus during the past 8 years the biological test, applied in the diagnosis of rabies here, has proved positive on ninety occasions, but in respect of only five viruses has the incubation period been less than 15 days, viz. 8, 11, 12, 14, 14; in the others it has varied between 15 and 28 days.

(b) *Fixation time.*

Mutation of street virus into fixed virus is normally accompanied by characteristic changes due to its progressive adaptation to the rabbit's nervous system. Reference has already been made to the fact that fixed virus is ordinarily obtained from the street virus of its origin only after some fifty passages through the rabbit, but Hoegyes (1900), for example, found that, when young rabbits were employed, a fixed virus could be achieved after about fifteen passages. Beham (1915), also, working in this country, effected fixation in respect of three street viruses after the fourth and sixth passages and reports have been made by many other workers concerning strains varying widely from the mean as regards rate of fixation. Indeed, if the suggestion of Levaditi and his colleagues (1924 *a*) were followed this difference in fixation-rate would constitute a satisfactory basis for classification of all street viruses and permit their separation into three main groups, viz.:

*Group I.* Highly virulent strains of street virus which show, even at the first passage, exceptionally short incubation periods and which, throughout all subsequent passages, retain their properties intact. Such viruses are termed "souches spontanément mutées" or "virus naturellement renforcé de Remlinger."

*Group II.* Less virulent strains in which mutation from street to fixed virus is easily effected but only after a certain number of passages in the rabbit. These viruses, referred to as "souches facilement mutables," cannot, despite ease of mutation, be included in the category of truly exalted strains.

*Group III.* Strains of low virulence in which change from street to fixed virus does not take place even after prolonged rabbit passage. These "souches non mutables" do, it is true, convey rabies to laboratory animals but only after an incubation period of irregular duration and of greater length than is experienced with any virus belonging to the first two groups.

In connection with this classification it appears to us desirable further to divide Group II into two sub-groups, viz.:

*Sub-group II (a).* Strains of abnormally high virulence in which complete fixation follows after but few passages.

*Sub-group II (b).* Strains of ordinary virulence in which fixation is completed after the average number of passages, i.e. about fifty.

We feel it all the more necessary to emphasise distinctions between street virus strains by such division and subdivision, as it is our considered opinion that a definite ratio exists between the degree of virulence and the fixation rate of rabies strains.

It is not proposed to discuss here Groups II (*b*) and III, which have little bearing on the subject under review; brief reference, however, must be made to certain typical strains in Groups I and II (*a*) if the use of fixation rate as a means of group differentiation is to be fully appreciated. Well-known viruses to be described under Group I are Tanger B (Chabrier), Puymyrol, Soucy and Koritschoner, under Group II (*a*) Gibraltar (Gréen), Tétuan, Tanger D (Martin) and Teodorascu. Of the Group II (*a*) strains, Gibraltar, Tétuan and Tanger D were first examined by Remlinger (1926) and then sent in glycerine to Levaditi for further investigation.

#### *Group I.*

(1) Moroccan strain—Tanger B (Chabrier) was originally derived from a young fox; rabbits first inoculated with the virus subdurally developed symptoms on the eighth day and died on the ninth, while in succeeding passages death occurred 7, 8, 7, 7, 8, 6, 6, 7, 7, 6, 7, 7, 7 days after inoculation. (Strain examined by Remlinger.)

(2) Paris strain—Puymyrol was recovered from a dog which had bitten his master on the day preceding its death; inoculated intramuscularly into a guinea-pig this virus caused death in 23 days; subsequent passages in rabbits showed periods between inoculation and death to be 8, 8, 8, 8, 9, 9, 8, 8, 8 days. (Strain examined by Levaditi.)

(3) Paris strain—Soucy was obtained from the brain of a dog diagnosed clinically rabid; guinea-pig infected intramuscularly died in 15 days; sub-passages in rabbits produced death 8, 8, 8, 8, 8, 8, 8, 9, 9 days after subdural inoculation. (Strain examined by Levaditi.)

(4) Koritschoner virus was first found in the brain of a man bitten by a rabid dog and dead of hydrophobia 24 days after completion of anti-rabies treatment (incubation period = 36 days); rabbits inoculated subdurally developed symptoms after 3 or 4 days' incubation and subsequent passages proved this interval between infection and appearance of symptoms to be constant. (Strain examined by Schweinburg (1925).)

#### *Group II (a).*

(1) Gibraltar strain (Gréen) was isolated from a dog which had, while showing signs of furious rabies, bitten three persons and a number of animals; first rabbit passage showed symptoms to follow subdural inoculation after 12 days, and death occurred in 14; death followed further rabbit subpassaging in 20, 11, 9, 10, 10, 8, 7, 8, 8, 8, 8 days and fixation was thus effected after the seventh passage.

(2) Moroccan strain—Tétuan was obtained from a dog dead of furious rabies; brain emulsions introduced into the anterior chamber of a rabbit's

eye caused the animal's death in 13 days; later research proved the intervals between subdural infection of rabbits and their death to be successively 10, 10, 11, 9, 12, 8, 10, 9, 8, 7 days; fixation took place here between the sixth and tenth passages.

(3) Moroccan strain—Tangier D (Martin) was derived from a basset hound, which had bitten many human beings and animals; subdural inoculation of a rabbit with bulb suspensions caused symptoms in 12 days and death in 14; subsequent passages caused death in 14, 11, 10, 9, 9, 10, 9, 9, 8, 9, 9, 9, 6 days; mutation of this virus began about the fourth passage and was complete at the eleventh.

(4) Teodorascu's virus was found in the brains of four people who, despite intensive anti-rabies treatment, had died of hydrophobia during the first 13 days following completion of the course (Puscariu III and Hoegyes I). Emulsions of brain matter taken from two of the cases were introduced into laboratory animals, killing guinea-pigs between the seventh and twentieth days and rabbits between the fourteenth and seventeenth days after subdural inoculation but producing no symptoms after subcutaneous infection. After the fifth intracranial passage through guinea-pigs, however, the virus became fixed with a constant incubation period of from 5 to 6 days, and it has since preserved a uniform virulence for rabbits, killing invariably between the seventh and tenth days.

Study of these strains amply shows that certain street viruses (Group I) behave from their first subpassages as fixed viruses and that thereby they can be sharply distinguished from a less exalted type—Group II (*a*), in which mutation is complete only after several passages in the rabbit.

(*c*) *Absence of Negri bodies.*

Mutation of street virus is normally accompanied by considerable modifications not only of its biological properties but also of its morphological characteristics. Negri bodies, for example, found regularly in the hippocampi of all creatures dead of street virus rabies, are hardly ever present in fixed virus infections. Absence of Negri bodies is, therefore, an important distinguishing feature between fixed virus and the street virus from which it is derived. The extent, then, to which Negri bodies proved to be present in the brains of rabbits infected with the strains just considered as typifying Groups I and II (*a*) is obviously germane to the present discussion and will now, therefore, be considered in brief. In Group I viruses Tangier B (Chabrier), Puymyrol and Soucy showed alike, from the first rabbit transfer, a complete absence of Negri bodies in the usual sites or at most a very rare occurrence of atypical forms. The Koritschoner virus, also, was wholly unable to effect Negri body formation in the brain of man or of experimental animals in successive passages, although, in the hippocampus of the dog, from which it was originally obtained, these bodies were present in exceptionally large numbers. Not only, therefore, in degree of infectivity and in brevity of incu-

bation period do naturally reinforced street viruses approximate to fixed virus but also in their loss of Negri body-producing power.

In Group II (*a*) the Gibraltar strain, as has already been shown, required for fixation seven passages in the rabbit; at the third passage Negri bodies appeared in the hippocampus and cerebral cortex to the extent of 80 per cent.<sup>1</sup>, at the fourth this figure had dropped to 10, at the sixth to 3 and at the seventh to zero; complete fixation and absence of Negri bodies were thus achieved at precisely the same passage. Tétuan virus, which proves fixation to have taken place between the sixth and tenth passages, showed an 80 per cent. incidence of Negri bodies between the second and fifth passages, but, from the sixth passage onwards the time between rabbit inoculation and death became shorter and more regular (7–9 days) and Negri bodies were altogether absent or only very occasionally present in an undeveloped form. In the Tangier D (Martin) strain fixation was finally secured at the eleventh passage, at a time when Negri bodies, previously present to the extent of 70–80 per cent., disappeared entirely from the brains of inoculated rabbits and when the interval between subdural infection and death had decreased to 7–9 days. As regards Teodorascu's virus, no information about Negri bodies is, unfortunately, available.

It is clear from an examination of Group II (*a*) strains that the virulent street viruses in this category do not become fixed at the first rabbit passage (as is the case with Group I strains) but give evidence of rapid mutation by a shortening of incubation period, by a greater regularity in the interval between inoculation and death and by a progressive decrease in the number and size of Negri bodies towards final disappearance; first total absence of Negri bodies synchronises, be it noted, with complete fixation of virus.

With regard to this subject it is unhappily the case that, while the research work of Williams and Lowden (1906), Negri (1909), J. Koch and Rissling (1910), Manouélian (1912), Watson (1913), Manouélian and Viala (1924) and Levaditi, Nicolau and Schoen (1924 *a*, 1924 *b*, 1926) has thrown considerable light on the nature and mode of transmission of rabies virus as well as on the significance of the Negri body, the International Rabies Conference of 1927 was unable from available evidence to decide whether the virus was a protozoon or a bacterium or whether Negri bodies represent a stage in the evolution of a living microsporidium or are produced by cellular degeneration. A plausible explanation, however, of the differences in Negri body content of brains infected with fixed and street viruses in general is to be found in the theory supported by many authors, that the rabies virus is protozoal in nature, belonging to the *Microsporidiida*, sp. *Glugea lyssae*. According to this conception the microparasite has a two-phase life history, one a phase of filtrable spores (only just, if at all, within the limits of visibility = Koch's

<sup>1</sup> The method adopted by Levaditi in his percentage estimation of Negri bodies was to note their occurrence in thirty-five pyramidal cells in the right Ammon's horn, thirty-five in the left horn and thirty in the cerebral cortex.



granulations) and of ultramicroscopic germs, the other a phase of intracellular sporoblastic encystment (Negri bodies). Fixed virus, on the one hand, consists solely of elementary and ultramicroscopic organisms in which reproduction occurs so rapidly that death of the infected animal is caused before the microsporidium has had time to complete its entire complex cycle of development and to arrive at the formation of the spherical uninucleate cells, pansporoblasts or sporonts considered to be Negri bodies. In street virus, on the other hand, where ordinarily development proceeds much more slowly, the survival of the infected animal together with the resistance offered to the virus by certain neurons, which have preserved their anatomical and physiological integrity, will permit the completion of the entire evolutionary cycle and consequently result in the appearance of the characteristic pansporoblasts. But in certain strains of street virus, in those "fixed at once," i.e. virus de rue renforcé, this distinction no longer holds, for, on account of the extraordinary virulence of these viruses, incubation period and interval to death are so short that, just as in fixed virus, there is no time for the pansporoblastic stage to be reached. (It has to be understood that this explanation is "based only on external analogies, that up to the present the whole developmental cycle (of the parasite) has not been sufficiently demonstrated and that, therefore, this theory, however attractive it may be, cannot claim to be anything more than a working hypothesis" (Lubinski and Prausnitz, 1926).)

## 5. DEFINITION.

From a consideration of the foregoing we suggest that virus de rue renforcé should be defined as a naturally exalted street virus, characterised by high degree of infectivity, by abnormally short incubation period and duration of illness, by complete mutation or fixation at the first passage in rabbits and by a behaviour thereafter in all respects akin to fixed virus, including an inability to effect Negri body formation.

The Safad virus, therefore, subscribing as it does to all these criteria, must be included in the category of such viruses.

## II. CROSS-IMMUNITY EXPERIMENTS.

### 1. INTRODUCTION.

Among the resolutions adopted at the International Rabies Conference held in Paris during 1927 was one to the effect that, *inter alia*, "enquiries should be made into the plurality of street virus strains." The need for such systematic investigation was apparent in view of the conflicting opinions expressed by leading authorities on the causes underlying abnormal behaviour of certain strains; thus by some, differences in "power of aggression" were explained on purely biological grounds, by others solely by specific variations in degree of virulence. That street viruses vary in individual power of attack is unquestionable; the observations of Bouffard (1912) and Heckenroth (1918), for example, on the occurrence in Senegal of a disease known as mad-dog

sickness or oulou-fato, which is in all respects similar to the rabies of Europe but which appears to be non-transmissible to man, instance in strains the nadir of virulence, while at the zenith are the naturally exalted viruses just considered. But at first sight this problem of plurality of strains appears capable of easy solution; theoretically submission of "atypical" viruses to the test of cross-immunity and to the action of the sera of immunised animals should place the matter beyond all doubt; in practice, however, results have been widely divergent. Thus Puntoni (1923) found that rabbits did not develop rabies if, during the period of ordinary incubation, they were given by the intravenous route carbolised suspensions of the street virus used for their subdural infection; in fact the only survivors proved to be those inoculated with homologous viruses and vaccines. Further, in a second set of experiments he tried the effect on fixed virus and on street virus of the serum of a human case treated with carbolised fixed virus and mixtures were prepared containing the viruses under test in 1 per cent. suspensions and 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:5000 dilutions of immune serum. For fixed virus a 1:500 dilution of serum was found to be rabicidal, but in the case of street virus 1:200 was required. These results led Puntoni to assume the biological individuality of certain strains of street virus and to raise the question of the expediency of polyvalent anti-rabies vaccines in treatment. Additional arguments in favour of the plurality of strains have since then been advanced by Calderini (1928) and by Teodorascu (1929). The former, in his examination of many street viruses, discovered one particular bovine strain, which differed markedly in its degree of virulence from all others examined. Normally even after many subpassages Calderini's strains were unable to cause the death of guinea-pigs in less than 5 days, but the bovine virus after thirty-five passages exhibited a degree of virulence sufficient to kill without exception on the fourth day. This hyper-virulence or exaltation of strain remained constant throughout twenty-six further passages through guinea-pigs and was not, in the author's opinion, the result of continued subpassage but was entirely dependent on some inherent character originally present while in the cow. Teodorascu makes use of the hypothesis of plurality of strains to account for four failures of treatment with monovalent vaccines. In support of this contention he refers to the results of his experimental work, viz. that treatment with ordinary fixed virus developed no immunity against his strains of street virus and, conversely, that vaccination with his strains afforded no protection against ordinary fixed virus when tested on rabbits. Again, the occurrence in Equatorial Africa of the practically avirulent viruses described by Bouffard and Heckenroth is held by some to constitute reason for the employment of polyvalent vaccines in the treatment of man. In direct opposition to these views, are the opinions expressed by Remlinger and his co-workers. Thus in discussing the viruses of Senegal, Remlinger (1928), while admitting an appreciable difference between the symptomatology of natural rabies in tropical countries and that encountered in more temperate zones—

the paralytic form largely predominates in the former—considers such distinction to indicate a geographical but not a radical difference in the strains. Further, by means of cross immunity and inoculation tests, Remlinger and Curasson (1924) claim to have established the identity of the oulou-fato virus with the naturally exalted Tangier B (Chabrier) strain and therefore with the virus of rabies itself. Again, Remlinger and Bailly (1930) investigated on similar lines a street virus, which appeared to be atypical in having, despite treatment, caused the death of two bitten persons; in as much as, according to their findings, the strain was indistinguishable by serological and immunity tests from a classical rabies virus, they concluded that the apparent difference was due to the specific power of activity and aggression possessed by the strains under review and to that power alone. According to this conception, therefore, the strains were biologically identical and the authors felt justified in pronouncing that “from the practical standpoint, whatever the virus may be, whether attenuated or exalted, polyvalent and auto-vaccines constitute a needless complication of anti-rabies treatment.”

Under the circumstances, then, it seemed to us desirable to institute enquiry into the behaviour of Safad virus in cross-immunity experiments and thereby to throw light, if possible, upon this vexed question, which is of paramount importance in anti-rabies immunisation.

## 2. EXPERIMENTAL EVIDENCE OF CROSS IMMUNITY FOLLOWING TREATMENT WITH CARBOLISED SUSPENSIONS OF PARIS (STANDARD) AND SAFAD (ATYPICAL) STRAINS OF RABIES VIRUS.

Degree of anti-rabies immunity possessed by rabbits actively immunised against rabies virus is ordinarily measured by their acquired resistance to infection with rabies strains and by an estimation of the rabicidal antibody content of their immune sera; should, then, any apparently atypical strain be encountered, its actions and reactions must surely be investigated along similar lines and compared with those of a classical virus. Moreover, this method of research obviously permits the following two arguments to be advanced regarding the question of “unity” or “plurality” of rabies virus, viz. (a) if, when tested by later subdural infections, the immunity following the exhibition of a classical virus in vaccine form is sufficient to confer on rabbits protection against homologous strain and atypical virus alike—and *vice versa*—proof of relationship between the two viruses is at once established; (b) if the classical strain is neutralised *in vitro* by the serum of rabbits immunised with the atypical virus and, conversely, if the atypical virus is neutralised under similar conditions by the serum of rabbits immunised with the classical strain, proof of actual identity now exists and the two viruses, whatever their differences in original virulence, cannot be separated on biological grounds.

The Safad virus at its twentieth subpassage in rabbits has, therefore, been compared in these respects with the Paris strain of fixed virus, it being

remembered that the Safad virus was "fixed" from the first rabbit transfer and that, in any case, fixed viruses, as far as crossed serum reactions are concerned, "act as the street viruses from which they are derived" (Marie, 1927).

This investigation required the employment of twenty rabbits whose sera had been proved naturally free from rabicidal properties. These rabbits were divided into four series, *A*, *B*, *C* and *D*, each series consisting of five rabbits. Each rabbit in Series *A* and *B* was treated for 14 days with 5 c.c. daily of a 2 per cent. suspension of killed carbolised Paris virus, and each rabbit in Series *C* and *D* received an identical dosage of killed carbolised Safad virus during the same period. The following experiments were then performed:

(a) Absolute immunity was tested in each rabbit series 52 days after completion of treatment, at a time when our previously recorded work (1929) has shown immunity to be at a maximum. Cross-immunity tests were carried out by the subdural infection of rabbits in each series with 0.2 c.c. of a 1:1000 suspension of fixed virus, viz. Series *A* and *C* were inoculated with Paris F.v., Series *B* and *D* with Safad F.v. The results are shown in Table III.

(b) Rabicidal antibody content was estimated in the immune sera of the treated rabbits. All rabbits were bled on the thirtieth day after completion of treatment and the sera of Series *A* and *B* and of Series *C* and *D* pooled separately. One unit volume of immune serum (0.5 c.c.) was then mixed with quantities of a 1:100 fixed virus, varying in each case from 0.5 c.c. to 3 c.c., and each mixture exposed to a temperature of 37° C. for 2 hours. At the end of this time 0.2 c.c. of each mixture was introduced subdurally into rabbits. By these means the rabicidal properties of Series *A* and *C* immune sera were examined in respect of Paris fixed virus, those of Series *B* and *D* immune sera in respect of Safad fixed virus.

From the results given in Table III it follows that Paris fixed virus, when used as immunising agent, was able to protect five out of five rabbits infected with living Paris fixed virus (100 per cent.) and three out of five rabbits infected with living Safad fixed virus (60 per cent.). Safad fixed virus, on the other hand, when used as immunising agent, was able to protect only one out of five rabbits infected with living Paris fixed virus (20 per cent.) and two out of five rabbits infected with living Safad fixed virus (40 per cent.).

Study of Table IV will show that: (a) One unit volume of the undiluted serum of rabbits immunised with killed carbolised Paris fixed virus and tested 30 days after completion of treatment was capable of neutralising 6 volumes of a 1:100 dilution of fresh living Paris fixed virus (homologous strain) and 4 volumes of fresh living Safad fixed virus (heterologous strain) in equal dilution. (b) One unit volume of the undiluted serum of rabbits immunised with killed carbolised Safad fixed virus and tested under identical conditions was capable of neutralising 2 volumes of a 1:100 dilution of fresh living Safad fixed virus (homologous strain) and 1 volume of fresh living Paris fixed virus (heterologous strain) in equal dilution.

Table III. *Immunity tests.*

The immunising power in rabbits of Paris and Safad fixed virus compared.

Rabbit series	Rabbit No.	Method of immunisation	Method, dose and time of infection with test dose	Result	Rabbit series	Rabbit No.	Method of immunisation	Method, dose and time of infection with test dose	Result
A	1	2% suspension	0.2 c.c. of a 1:1000	Lived	B	1	2% suspension	0.2 c.c. of a 1:1000	Lived
	2	of Paris f.v.:	suspension of Paris	Lived		2	of Paris f.v.:	suspension of Safad	Lived
	3	5 c.c. daily for	f.v. subdurally, 52	Lived		3	5 c.c. daily for	f.v. subdurally, 52	Died*
	4	14 days	days after end of	Lived		4	14 days	days after end of	Died
	5		treatment	Lived		5		treatment	Lived
C	1	2% suspension	0.2 c.c. of a 1:1000	Died	D	1	2% suspension	0.2 c.c. of a 1:1000	Lived
	2	of Safad f.v.:	suspension of Paris	Lived		2	of Safad f.v.:	suspension of Safad	Died
	3	5 c.c. daily for	f.v. subdurally, 52	Died		3	5 c.c. daily for	f.v. subdurally, 52	Died
	4	14 days	days after end of	Died		4	14 days	days after end of	Lived
	5		treatment	Died		5		treatment	Died*
Control	1	Nil	0.2 c.c. of a 1:1000	Died	Control	1	Nil	0.2 c.c. of a 1:1000	Died
	2		suspension of Paris	Died		2		suspension of Safad	Died
	3		f.v. subdurally	Died		3		f.v. subdurally	Died

\* Incubation period prolonged.

Table IV. *Estimation of rabicidal antibody content in immune sera.*

Rabicidal properties possessed by the sera of rabbits immunised with Paris and with Safad fixed virus respectively.

Rabbit Series A and B: immunised with a 2% emulsion of killed carbolised Paris virus in a dosage of 5 c.c. daily on 14 consecutive days.

Rabbit Series C and D: immunised with a 2% emulsion of killed carbolised Safad virus in a dosage of 5 c.c. daily on 14 consecutive days.

Immune serum	Rabbit No.	Mixtures of immune serum and varying amounts of living fixed virus		Result of subdural inoculation of rabbits with 0.2 c.c.	Immune serum	Rabbit No.	Mixtures of immune serum and varying amounts of living fixed virus		Result of subdural inoculation of rabbits with 0.2 c.c.
		Amount of serum in c.c.	Amount of 1:100 Paris f.v. in c.c.				Amount of serum in c.c.	Amount of 1:100 Safad f.v. in c.c.	
Series A (Paris)	1	0.5	0.5	Lived	Series B (Paris)	1	0.5	0.5	Lived
	2	0.5	0.5	Lived		2	0.5	0.5	Lived
	3	0.5	1.0	Lived		3	0.5	1.0	Lived
	4	0.5	1.0	Lived		4	0.5	1.0	Lived
	5	0.5	2.0	Lived		5	0.5	2.0	Lived
	6	0.5	2.0	Lived		6	0.5	2.0	Died
	7	0.5	3.0	Lived		7	0.5	3.0	Died
	8	0.5	3.0	Lived		8	0.5	3.0	Died
Series C (Safad)	1	0.5	0.5	Lived	Series D (Safad)	1	0.5	0.5	Lived
	2	0.5	0.5	Lived		2	0.5	0.5	Lived
	3	0.5	1.0	Died		3	0.5	1.0	Lived
	4	0.5	1.0	Died		4	0.5	1.0	Lived
	5	0.5	2.0	Died		5	0.5	2.0	Died
	6	0.5	2.0	Died		6	0.5	2.0	Died
	7	0.5	3.0	Died		7	0.5	3.0	Died
	8	0.5	3.0	Died		8	0.5	3.0	Died
Controls not immunised	1	0.5	0.5	Died	Controls not immunised	1	0.5	0.5	Died
	2	0.5	1.0	Died		2	0.5	1.0	Died
	3	0.5	1.5	Died		3	0.5	1.5	Died

## 3. DISCUSSION.

A combination of the results given in Tables III and IV permits an interesting comparison to be made between Paris (classical) fixed virus and Safad (atypical) fixed virus in respect of their immunising and rabicidal antibody producing powers—a comparison which has a very definite bearing on the question of plurality of rabies strains.

In the first place it will be observed that the effect on rabbits of vaccination with Paris fixed virus was (a) to afford 100 per cent. protection against the homologous strain and 60 per cent. against the exalted or atypical strain,

and (b) to confer on the sera of rabbits so treated rabicidal properties capable of neutralising 6 unit volumes of a 1 per cent. suspension of the homologous virus and 4 volumes of the atypical strain. Secondly it is shown that the effect on rabbits of vaccination with Safad fixed virus was (a) to afford 40 per cent. protection against the homologous strain and 20 per cent. against the classical Paris strain, and (b) to confer on the sera of rabbits so treated rabicidal properties capable of neutralising 2 unit volumes of a 1 per cent. suspension of the homologous virus and 1 volume only of the classical strain. The results of the immunising experiments, then, clearly show that, whereas Paris fixed virus in the form of carbolised vaccine—the ordinary immunising agent used here in anti-rabies treatment—protected rabbits in a highly satisfactory way against subsequent infections with homologous and heterologous strains alike, the Safad fixed virus as vaccine compared unfavourably in its power of protecting rabbits against either virus used in the subdural test. Indeed, the resistance to fresh Safad virus offered by rabbits previously treated with Safad fixed virus vaccine fell considerably short of that shown by rabbits treated with ordinary Paris fixed virus vaccine, while the protection conferred by Safad fixed virus vaccine against subsequent infection with living Paris fixed virus was decidedly unsatisfactory. As an immunising agent, therefore, both in general and in particular, the Paris strain of fixed virus proved unquestionably far superior to the Safad strain. Treatment of the Safad cases with an autogenous vaccine would obviously then have served no useful purpose and it is further logical to assume that, if strain *X* be a virus endowed with adequate immunising power and strain *Y* be deficient in this respect, the use in anti-rabies treatment of strain *X* vaccine alone would be of greater service than a vaccine in which strain *X* and *Y* were combined. An argument is thus advanced in support of the continued use of a monovalent anti-rabies vaccine of proved immunising ability and against the need for preparation of polyvalent or autogenous vaccines.

Next with regard to the serum-neutralisation experiments, it is immediately apparent that anti-viral bodies were evoked in rabbits to a much greater extent by treatment with Paris virus than with Safad virus, quantitative estimation being based on the power possessed by immune sera of neutralising, volume by volume, centesimal fixed virus emulsions. Moreover, as a result of this investigation, a very definite, indeed an almost mathematical relationship was established in treated rabbits between their degree of acquired immunity and the rabicidal antibody content of their blood; thus when the percentage of acquired immunity was 100 (homologous), 60 (heterologous), 40 (homologous) and 20 (heterologous) the rabicidal power of the corresponding immune sera was respectively 6-, 4-, 2- and 1-fold. It is our belief that this remarkable concurrence is unlikely to arise from random causes or be accounted for by coincidence alone; on the contrary, despite the small number of rabbits employed, it would appear to be of certain significance: that, with the notable exception of those cases wherein solid immunity has

been recorded without corresponding anti-viral formation in the serum, rabicidal antibody content is ordinarily, if not identical with, at least indicative of anti-rabies immunity. If, now, this conclusion is allowed, it follows that a further reason has been advanced against the necessity of employing auto-genous or polyvalent vaccines in anti-rabies treatment.

To turn finally to the question of relationship between the two viruses under review. "To-day it is generally recognised that modifications in the activity of rabies virus strains can only be explained by the presence of a micro-organism" (Marie, 1927). The same criteria, therefore, must, where possible, be applied in the classification of strains of rabies virus and of bacteria. Now, of the several means available for determining the identity or non-identity of two bacteria, the power possessed by the bacterial body of calling forth in the blood of living animals substances or properties of an antagonistic nature is unquestionably the most delicate. The protein, protein-lipoid complex or other compound capable of acting as an antigen gives rise to its own special antibody and the reaction between antibody and antigen is of a highly specific character. Upon the degree of specificity, therefore, in immunological reactions will the establishment of intimate phylogenetic relationship between two bacteria or two rabies strains ultimately depend, and in the case of rabies virus such will be achieved by means of protective tests and serum-neutralisation experiments. A glance at Tables III and IV will show that, from the point of view both of cross-protection tests and crossed serological reactions, the classical (Paris) strain and the Safad (exalted) strain may, despite apparent differences, be safely included within the same category of rabies virus. True, a higher degree of protection was afforded to rabbits and the rabicidal antibody content in their sera was greater after treatment with Paris virus than with the Safad strain, but this can readily be explained by different immunising properties of the two viruses; moreover, that rabies viruses differ in this respect need occasion no more surprise than that certain strains of *Bact. typhosum* should be preferred in anti-enterica prophylaxis.

From a consideration of the above and in the light of our experience with these two strains, we are of opinion that exalted virus and classical virus are not to be differentiated by immunological tests and are, indeed, identical. Our views, therefore, support the conclusions of Remlinger and his co-workers and are in opposition to those expressed by Puntoni, Calderini and Teodorascu.

#### SUMMARY.

1. The occurrence of an exalted strain of rabies virus has been recorded and a definition given of virus de rue renforcé.
2. The characteristics of exalted virus have been examined in respect of incubation time, fixation rate and absence of Negri bodies.
3. An adequate classification of street virus strains has been suggested.
4. Cross-immunity experiments have been carried out and, as a result of

cross-protection tests and of crossed serum reactions, identity of exalted virus with the classical strain has been established.

5. The employment of autogenous and polyvalent vaccines has been shown to be unnecessary in anti-rabies immunisation.

## REFERENCES.

- ABBA (1898). Statistique de l'Institut antirabique de Turin. *Ann. Inst. Pasteur*, **12**, 34.
- ALIVISATOS (1926). Neue Erfahrungen bei der Schutzimpfung gegen Lyssa durch das ätherisierte Virus fixe. *Zentralbl. Bakt.* **98**, 394.
- D'AMATO (1904). Sur l'existence dans la nature d'un virus rabique renforcé. *Riforma med.* **11**, 1.
- BABES (1912). *Traité de la rage*. Paris: Baillière et Fils.
- BAUER (1886). Inkubationsdauer der Tollwut beim Menschen. *München. med. Wochenschr.* **1**, 37-39.
- BEHAM (1915). Schnelle Verwandlung des Strassenvirus der Tollwut in Virus fixe. *Ibid.* **30**, 841.
- BOUFFARD (1912). Sur l'existence de la rage canine dans le Haut-Sénégal et le Niger. *Ann. Inst. Pasteur*, **38**, 258.
- BUSSON (1928). Charakteristik des Begriffes Virus fixe. *Wien. klin. Wochenschr.* **41**, 1145.
- CALABRESE (1896). Sur l'existence dans la nature d'un virus rabique renforcé. *Ann. Inst. Pasteur*, **10**, 97.
- CALDERINI (1928). Un caso di eccezionale esaltazione della virulenza del virus rabbico nella cavia. *Giorn. di Batteriol. e Immunol.* **3**, 429.
- HARVEY and ACTON (1923). The degree of efficacy of antirabic treatment. *Ind. J. Med. Res.* **9**, 868.
- HARVEY and McKENDRICK (1930). "Rabies" in *A System of Bacteriology*. London: H.M. Stationery Office.
- HECKENROTH (1918). Contribution à l'étude de la rage en Afrique occidentale française. *Ann. Inst. Pasteur*, **32**, 389.
- HELMAN (1888). Étude sur les formes furieuses et paralytiques de la rage chez le lapin. *Ibid.* **2**, 274.
- HOEGYES (1900). "Lyssa" in Nothnagel's *Spezielle Pathologie und Therapie*. Vienna.
- KOCH, J. (1929). Kann die Differentialdiagnose, ob der Tod eines während oder nach der Pasteurschen Schutzimpfung gestorbenen Patienten durch das Strassen- oder Passagevirus verursacht wurde, durch den Tierversuch gestellt werden? *Zentralbl. Bakt.* **113**, 376.
- KOCH and RISSLING (1910). Studien zur Aetiologie der Tollwut. *Zeitschr. Hyg. InfektKr.* **65**, 85.
- KONRADI (1923). Auffallend kurze Inkubationen bei menschlicher Wut. *Zentralbl. Bakt.* **90**, 162.
- LEVADITI, NICOLAU and SCHOEN (1924 a). La nature du virus rabique. *C.R. Soc. Biol., Paris*, **90**, 994.
- — — (1924 b). La nature microsporidienne du virus rabique. *Ibid.* **90**, 398.
- — — (1926). Recherches sur la rage. *Ann. Inst. Pasteur*, **40**, 973.
- LUBINSKI and PRAUSNITZ (1926). Lyssa. *Ergebn. Hyg. Bakt.* **8**, 1.
- McKENDRICK (1929). Rabies. A review of recent articles. *Trop. Dis. Bull.* **26**, 216.
- MANOUÉLIAN (1912). Étude des corpuscules de Negri et des formations spéciales à la rage à virus fixe. *Ann. Inst. Pasteur*, **26**, 973.
- MANOUÉLIAN and VIALA (1924). "Encephalitozoon rabiei" parasite de la rage. *Ibid.* **38**, 258.
- MARIE (1927). La nature du virus rabique. *Report to International Rabies Conference*.



- NĖGRI (1909). Ueber die Morphologie und den Entwicklungszyklus des Parasiten der Tollwut. *Zeitschr. Hyg. InfektKr.* **63**, 421.
- PUNTONI (1923). La pluralità biologica del virus rabico da strada. *Ann. Igiene (sper.)*, **33**, 1.
- REMLINGER (1908). La rage chez les tout jeunes chiens. *C.R. Soc. Biol., Paris*, **60**, 508.
- (1924). Sur un virus renforcé recueilli au Maroc. *Bull. Soc. Path. exotique*, **17**, 538.
- (1926). Note sur les virus de rue envoyés de Tanger à M. Levaditi. *Ann. Inst. Pasteur*, **40**, 1070.
- (1928). Quelques considérations sur la rage et le traitement antirabique dans les pays tropicaux. *Bull. Soc. Path. exotique*, **21**, 790.
- REMLINGER and BAILLY (1930). Unicité ou pluralité du virus rabique. *Bull. Acad. Méd., Paris*, **103**, 408.
- REMLINGER and CURASSON (1924). Identité de l'oulou-fato et de la rage. *Ibid.* **92**, 112.
- SCHWEINBURG (1925). Ueber das Virus Koritschoner. *Zeitschr. Immunitätsforsch.* **42**, 552.
- (1928). Zur Frage der Virulenz des Virus fixe. *Wien. klin. Wochenschr.* **41**, 1149.
- STUART and KRIKORIAN (1929). Studies in antirabies immunisation. *J. Hyg., Camb.* **29**, 1-34.
- TEODORASCU (1929). Études expérimentales sur la virulence particulière de deux souches de virus rabique. *C.R. Soc. Biol., Paris*, **100**, 929.
- WATSON (1913). The Negri bodies in rabies. *J. Exp. Med.* **17**, 29.
- WILLIAMS and LOWDEN (1906). The etiology and diagnosis of hydrophobia. *J. Infect. Dis.* **3**, 452.

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# POTATO STARCH AND REFECTION.

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(With one Text-figure and one Chart.)

SINCE Fridericia (1926) first reported the spontaneous appearance of refection in rats of his colony receiving vitamin B-deficient diets the phenomenon has been observed by various investigators and the accuracy of Fridericia's findings amply confirmed (Fridericia, Freudenthal, Gudjonsson, Johansen and Schoubye, 1927; Roscoe, 1927; Kon and Watchorn, 1928; Scheunert, Schieblich and Rodenkirchen, 1929; Schieblich and Rodenkirchen, 1929, 1, 2).

Taylor and Thant (1929) have reported refection in pigeons, while Bechdel, Eckles and Palmer (1926), Bechdel and Honeywell (1927) and Bechdel, Honeywell, Dutcher and Knutsen (1927, 1928) have shown that ruminants thrive on diets found to be deficient in vitamin B by experiments on rats and have isolated from the rumen of a cow subsisting on a vitamin B-deficient diet an organism, *Flavobacterium vitarumen*, considered by them to be responsible for the endogenous synthesis of vitamin B.

It is only fair to state that, as early as 1915, Theiler, Green and Viljoen (1915) advanced the hypothesis that vitamin B is synthesised in the alimentary tract of ruminants.

In 1929 Mendel and Vickery (1929) reported that they were unable to observe in their laboratories phenomena comparable to refection either by using diets containing raw maize starch or dietaries similar to those used by Kon and Watchorn (1928) containing potato starch prepared in the laboratory from potatoes. The results obtained by the former authors when raw potato starch was used will be discussed in a later part of this paper.

The criticism by Mendel caused me to repeat the experiments with raw potato starch. This was done in the laboratory from which the present work is published. Previously I also produced refection with diets containing raw potato starch in the laboratory of Prof. H. M. Evans during my visit to California in 1928. As the actual figures obtained there are not available this set of experiments will not be discussed further and only a photograph showing two littermate rats which had been kept for some time on the refection diet deficient in vitamin B will be included. One of the animals became relected while the other showed the usual signs of vitamin B deficiency.

As a result of my work I can fully confirm the previous findings of Kon and Watchorn (1928) that refection becomes almost the rule when raw potato starch is used.

## EXPERIMENTAL.

*Raw potato starch diet.* Seven young albino rats weighing from 49 to 77 gm. were placed on October 18th, 1929, on the following diet:

Casein (washed for a week with acidulated water)...	23 per cent.
Potato starch (commercial, moisture 14.2 per cent., N 0.018 per cent.) ... ..	57 "
Hardened vegetable fat ("Ceres") ... ..	15 "
Salt mixture (Steenbock's, No. 40) ... ..	5 "
Cod-liver oil: 2 drops per rat per day.	

The rats were kept together in a cage (dimensions  $18 \times 10 \times 9$  in.) on a half-inch mesh screen. The diet and tap water were offered *ad lib*. The growth curves of the rats are given in Chart 1.



Fig. 1. Two littermate rats kept on raw potato starch diet. One became refeed while the other showed the usual signs of vitamin B deficiency.

*Appearance of the rats.* Shortly after the beginning of the experiment all the rats were in bad condition: their fur was very ruffled, and for the first 10 days the weight was either stationary or dropped a little. One rat (1080) died after 5 days. On autopsy a very severe meteorism was found, the intestines were enormously distended with gas and the mesenteric vessels injected with blood. The lungs were atelectatic owing to the pressure of the abdominal contents. At the end of the second week the remaining rats started growing and their fur became smoother and better though by no means normal. From now on four rats (1077, 1078, 1081 and 1083) grew steadily at a subnormal rate. Two rats, 1079 and 1082, after a preliminary period of growth declined and died in the course of 82 and 81 days respectively without showing definite symptoms of vitamin B deficiency other than emaciation, weakness and roughness of coat.

Rat 1081 became pregnant 5 months after the beginning of the experiment and gave birth to one normal stillborn pup and seven foetuses partly macerated. At the same time a vaginal plug containing spermatozoa was found in female 1083, but no litter was born to her.

On January 15th, 1930, three new rats, Nos. 1146 and 1147, 22 days old,

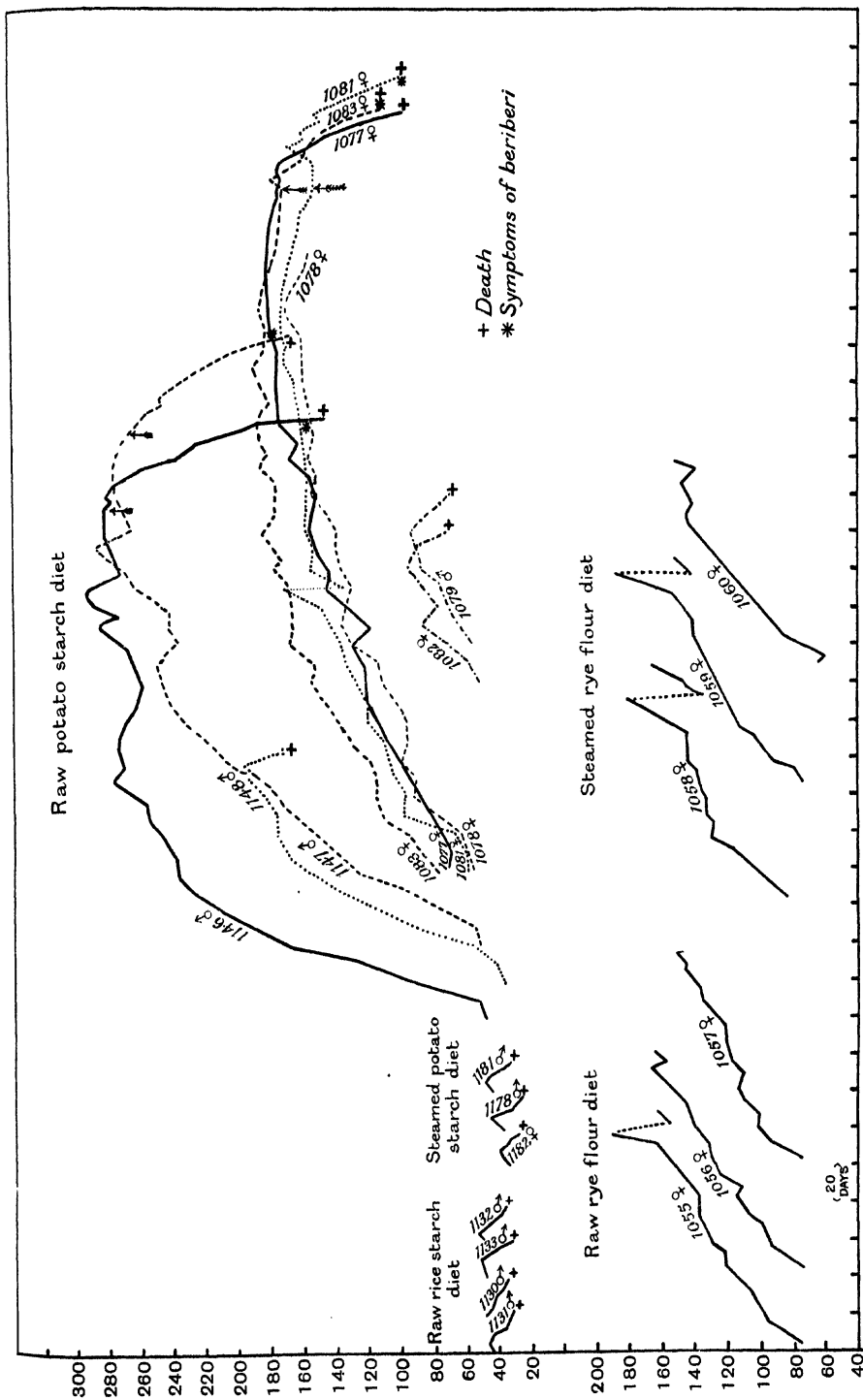


Chart I.

and 1148, 21 days old, were placed in the same cage. Within a week all three started growing at a rapid rate, rat 1146 for example making the following weekly gains:—2, 19, 28, 25, 25, 25, 23, 13, 10 and 15 gm., by which time he had reached a weight of 225 gm. It then progressed more slowly to a weight of almost 300 gm. These rats had good sleek fur, were very lively and seemed absolutely normal. They were much better in appearance than the rats first placed on experiment. Rat 1147, after having attained a weight of 190 gm. after 4 months on the diet, suddenly declined and died.

Rat 1078 developed a large abscess on the lower jaw, causing him much discomfort, and was killed on September 13th, 1930, after it had been almost 11 months on the raw potato starch diet.

*Change of diet.* On October 18th, 1930, exactly 1 year after the commencement of the experiment a change was made in the diet. From that date, instead of being offered raw, the diet was made up to a paste with water and steamed on the water bath with constant stirring for 3 to 5 minutes. The starch had then dextrinised and the whole mass set to a jelly. The change of diet is marked by arrows on the graphs. As a result all rats declined and died within 2 months (rat 1077 after 35 days, rat 1081 after 58 days, rat 1083 after 41 days, rat 1146 after 48 days and rat 1148 after 49 days). Rats 1081, 1083, 1146 and 1148 showed typical symptoms of beriberi: convulsions, incoordination and spasticity. After the change of diet rats 1077, 1081, 1083 and 1148 also showed progressive symptoms generally considered to be characteristic of vitamin B<sub>2</sub> deficiency, *i.e.* loss of hair, blood on the paws and vibrissae, and also urine-stained abdomens, while rat 1081 developed in addition swollen and reddened eyelids.

*Steamed potato starch diet.* Three young rats, Nos. 1178, 1181 and 1182, weighing 36, 42 and 31 gm. respectively, were placed on the steamed potato starch diet. They grew for a short time, then rapidly declined and died within 3 weeks without showing any definite symptoms. The growth curves are given in Chart I.

*Rice starch diet.* Four young rats, Nos. 1130, 1131, 1132 and 1133, weighing from 40 to 50 gm., were placed on the following diet:

Casein (washed for a week with acidulated water) ...	23 per cent.
Rice starch (commercial, moisture 13.1 per cent., N 0.073 per cent.) ... ..	57 „
Hardened vegetable fat ("Ceres") ... ..	15 „
Salt mixture (Steenbock's, No. 40) ... ..	5 „
Cod-liver oil: 2 drops per rat per day.	

The rats in this experiment (as well as in the preceding experiment) were kept together in a cage of the same type as used for the rats on the raw potato starch diet. They rapidly declined and died within 3 weeks. The growth curves (Chart I) are quite similar to the growth curves of rats on steamed potato starch diet.

*Stability of the vitamin B complex in steamed diets.* Three young female rats were placed on the following diet:

Bleached rye flour	...	...	...	...	...	88 per cent.
Butter fat	...	...	...	...	...	9 „
Salt mixture (Steenbock's, No. 40)	...	...	...	...	...	3 „

This diet had previously been shown (unpublished experiments) to contain inadequate amounts of the vitamin B complex permitting only subnormal growth at about the same rate as that observed in the first group of rats on the potato starch diet.

Three other females received this diet which had been steamed under precisely the same conditions as described previously for the potato starch diet. There was no noticeable difference between the two groups of animals. The reproductive cycles were very irregular in both groups. When exhibiting signs of oestrus the females were mated with normal males from the breeding colony. One female from the raw diet group and two from the steamed diet group had litters. A large percentage of the young were born dead. None were suckled and all were eventually destroyed by the mothers. The growth curves of both groups are given in Chart I.

*Faeces of refected rats.* At frequent intervals freshly voided faeces were collected separately from the rats on the potato starch diet, and observed for colour, size and presence of starch by iodine staining. The results confirm the opinion of Schieblisch and Rodenkirchen (1929, 1) that refection may be present without the faeces becoming white or especially bulky. In fact typical white faeces were observed only occasionally in this experiment and not always in those rats showing the best growth. As a rule they were already dark when voided or had a creamy colour immediately after which darkened rapidly on drying. The size also fluctuated quite markedly, the faeces being at times fairly small and of irregular shape. No check was noticed at such times in the growth or general appearance of the refected rats. Unbroken starch granules were always present.

*Bacterial flora.* Examinations of the bacterial flora have been made and it is hoped to report in a separate publication the results of a bacteriological examination of the faeces.

#### DISCUSSION.

Mendel and Vickery (1929) believe that they have failed to observe in their laboratories phenomena comparable to refection. It is significant, however, that while all four of their rats placed on a maize starch diet declined and died in the usual way, two out of six rats fed on a potato starch diet behaved abnormally. One of them (rat B 6963) especially at first grew and then maintained its weight for almost 4 months. The explanation is advanced by Mendel and Vickery that potato starch at times may carry traces of vitamin B, while Dr Gudjonsson of Prof. Fridericia's laboratory who visited

those workers at the time of the experiment expressed the opinion that there was no evidence of refection in the case of rat B 6963 (Mendel and Vickery, 1929).

If vitamin B is present in potato starch one would expect the whole group of rats receiving the potato starch diet to behave similarly, especially as the starch was prepared in the laboratory under well-controlled conditions. On the other hand, if refection is caused by an endogenous bacterial agent (and there is at present no better, or simpler explanation of the phenomenon) then wide variations in the behaviour of rats submitted to conditions likely to produce refection would naturally be expected. Rats will thrive or die on refection diets according to the degree of susceptibility, exhibiting all stages of nutritional condition from a rapid decline and death, typical of vitamin B (complex) deficiency, through shorter or longer periods of maintenance, to instances of rapid growth, normal appearance and almost unlimited survival. An "all or nothing" law is certainly not applicable in the case of refection.

The present work shows an interesting example of variations in the degree of refection. The second group of rats did much better on the potato starch diet than the first group, while marked differences are noticeable within the groups. Most probably the better performance of the second group is due to a massive "infection" at an early stage of life contracted from refection individuals of the first group.

It is obvious that spontaneous refection is of quite common occurrence when potato starch is used. In my experience this is not the case with other starches. I have not observed spontaneous refection with rice starch, nor with diets containing varying proportions of raw wheat and rye flours. Growth and survival on diets containing raw potato starch are certainly not due to the presence of vitamin B (complex) in the starch. The refection ability of the starch is completely lost under conditions having no deleterious influence on the vitamin B complex present in other food materials. A comparison of the growth curves of rats receiving raw and cooked potato starch diets with those of rats fed on raw and cooked flour diets is sufficiently convincing. Moreover, even the presence of traces of vitamin B (complex) in the starch could not account for sustained growth at a rate of almost 4 gm. a day or for the survival of rats for periods covering more than one-third of the normal total span of life. The enormous variations within groups of rats receiving the raw potato starch diet also speaks against the presence of a fixed preformed amount of vitamin B (complex) in the diet and for an endogenous bacterial synthesis.

#### SUMMARY AND CONCLUSIONS.

1. Refection occurs as a rule in rats receiving diets containing raw potato starch.

2. Rats were maintained for one year on a diet deficient in the vitamin B complex and containing raw potato starch. Two of them attained a weight of almost 300 gm. and made sustained gains at a rate of almost 4 gm. daily.

3. Rapid decline, symptoms of vitamin B<sub>1</sub> and B<sub>2</sub> deficiency and death were observed in these rats after their diet had been steamed.

4. Steaming under identical conditions does not alter the vitamin B (complex) content of other diets.

## REFERENCES.

- BECHDEL, S. I., ECKLES, C. H. and PALMER, L. S. (1926). *J. Dairy Sci.* **9**, 409.  
BECHDEL, S. I. and HONEYWELL, H. E. (1927). *J. Agric. Res.* **35**, 283.  
BECHDEL, S. I., HONEYWELL, H. E., DUTCHER, R. A. and KNUTSEN, M. H. (1927). *Pennsylvania Agric. Exp. Sta. Bull.* No. 213, p. 19.  
BECHDEL, S. I., HONEYWELL, H. E., DUTCHER, R. A. and KNUTSEN, M. H. (1928). *J. Biol. Chem.* **80**, 231.  
FRIDERICIA, L. S. (1926). *Skand. Arch. f. Phys.* **49**. Abstracts of communications to 12th Internat. Physiol. Congress, p. 55.  
FRIDERICIA, L. S., FREUDENTHAL, S., GUDJONSSON, S., JOHANSEN, G. and SCHOUBYE, N. (1927). *J. Hygiene*, **27**, 70.  
KON, S. K. and WATCHORN, E. (1928). *Ibid.* **27**, 321.  
MENDEL, L. B. and VICKERY, H. B. (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 552.  
ROSCOE, M. H. (1927). *J. Hygiene*, **27**, 103.  
SCHIEBLICH, M. and RODENKIRCHEN, J. (1929, 1). *Biochem. Z.* **213**, 234.  
——— (1929, 2). *Ibid.* **213**, 245.  
SCHEUNERT, A., SCHIEBLICH, M. and RODENKIRCHEN, J. (1929). *Ibid.* **213**, 226.  
THEILER, A., GREEN, H. H. and VILJOEN, P. R. (1915). *3rd and 4th Reports, Director Veterinary Res. Dept. Agric. Union S. Africa.*  
TAYLOR, J. and THANT, U. (1929). *Indian J. Med. Res.* **16**, 747.

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# THE POISONOUS PRINCIPLE OF *LATHYRUS* AND SOME OTHER LEGUMINOUS SEEDS.

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(With Plate VII.)

SINCE remote times man has cultivated many kinds and varieties of peas and beans as food for himself and his domestic animals, and with two notable exceptions there has apparently been little suspicion on his part that they might be potentially poisonous or deleterious. The exceptions are the bitter vetch (*Ervum ervilia* L.) and the vetchling *Lathyrus* (*L. sativus*, *L. cicera* and *L. clymenum*), the former of which ever since Hippocrates recorded the first known epidemic and the latter since the seventeenth century have been recognised as liable to injure farm animals and to cause in man an incurable paralysis of the legs if eaten in too large quantity as part of the daily dietary. The bitter vetch is now only of limited agricultural importance as a cattle food, but lathyrus is extensively grown in France and southern Europe chiefly as fodder for farm stock and also to some extent for human consumption. The peasants cook and eat it like other pulses and the meal mixed with wheat or barley flour is made into bread, hence in times of scarcity and dear cereals an excessive use of it has often given rise to local outbreaks of poisoning. In India and in Kabylia the peas, whole or ground and cooked in various ways, form the staple diet of large sections of the poorer classes, as they are cheap, palatable, and very nutritious, and in times of famine the increased consumption has been the cause of many recorded pandemics. Apart from famines, however, large agricultural populations in some parts of India subsist to a great extent on lathyrus peas (khesari, teora, matra) and in certain States of North-West and Central India 6 per cent. of the total inhabitants are said to be affected in consequence by paralysis of the lower limbs, and in some of the worst villages 10 per cent. or more of the adult males. The condition has always been well known under various names to physicians practising in the countries affected, but in 1873 Cantani (Naples) gave it the name *Lathyrism* under which it is now usually described in text-books of medicine. As very large numbers of mankind habitually consume lathyrus peas it may safely be assumed that they are innocuous when taken as a moderate part of a mixed dietary, and Buchanan states that in some Indian jails 4-6 ounces per person have been given daily for years without any bad results having been observed, while on the other hand, Grandjean and others find that when they form almost the whole dietary paralysis supervenes in 4-8 weeks. But the grain

varies considerably in its content of toxic substance and the results depend partly on this, partly on the amount eaten, and probably to some extent on individual susceptibility. Men are much more liable to be affected than women, in the ratio of 10 or 12 to 1, and boys than girls. Slight cases of lathyrism show merely a certain degree of motor paralysis and spasticity in the lower limbs which may pass off. In more severe cases the paralysis comes on suddenly and may involve the bladder, rectum, and genital organs, while there may also be pains round the waist, lightning pains, loss of sensation, numbness, cramps, and prickling. All these symptoms, if present, usually clear up with the exception of the spastic paralysis which varies greatly in degree and is permanent. Chevallier (France 1841) mentions somnolence as a symptom, Brunelli, who reported eleven cases which occurred in the neighbourhood of Rome (1880), states that after each meal certain of them showed a kind of transient intoxication, and Desparanches, who described an extensive outbreak round about Blois (1829), says that convulsive movements of the limbs is the earliest symptom in some cases. It is evident therefore that the poison may affect any part of the brain and spinal cord, but that its chief and permanent effect is exercised on the motor tracts from the cortex downwards. The general health remains good, and it does not directly shorten life. Lt.-Col. McCombie Young has published a very clear analysis and explanation of the motor clinical symptoms which makes any further account of them here unnecessary<sup>1</sup>, and Proust's report on an outbreak in Kabylia also gives very full details<sup>2</sup>. No post-mortem examination of the nervous system in man has ever been made.

As regards the action on animals, the whole plant and the peas are largely used as fodder and the addition of 20 per cent. of the peas to other feeding stuffs is well known to be innocuous except to horses which are peculiarly susceptible. But even when fed entirely on the peas for long periods herbivora and pigs thrive and remain well although they are apt to develop a weakness in the hind legs. Pigs turned into fields of lathyrus to feed have not infrequently died of acute poisoning and sheep and cattle have also died acutely. Ducks, geese and peacocks are readily poisoned by the peas, but pigeons, hens and partridges do well on them although perhaps not quite immune. In a long series of feeding experiments with monkeys, I was able to produce most of the symptoms of lathyrism as seen in man, and by continuing the feeding to bring on more aggravated symptoms, sometimes with a fatal termination. The animals became very paretic and weak muscularly and suffered at irregular intervals from generalised spasmodic attacks in which they were almost completely helpless. Some of them died in one of these attacks, others recovered apparently completely, and others remained permanently paretic. For details and a more extended account of lathyrism I must refer to two previous communications<sup>3</sup>.

<sup>1</sup> *Indian J. Med. Research*, 1927, 15.

<sup>2</sup> *Bull. Acad. Méd.* 1883, 12, 829.

<sup>3</sup> *Edinb. Med. J.* Nov. 1917 and *J. Pharmacol. and Exper. Therap.* 1929, 37.

THE POISONOUS PRINCIPLE OF *L. SATIVUS* AND *L. CICERA*.

Both the small and large varieties of *L. sativus* peas grown in India and *L. cicera* peas grown in France were used, and no differences were noticed either in their action or chemistry.

The poisonous principle is an acid which may be extracted by macerating the ground peas in cold water or weak alcohol for several days, expressing, boiling the resulting liquid to precipitate albumen, filtering off the albumen and concentrating the liquid in a shallow pan at a very gentle temperature with the aid of a fan. It is very acid in reaction and may be further purified by precipitation with mercuric chloride, treating the filtrate from this with  $H_2S$ , filtering off the mercury sulphide, passing a current of air through the filtrate to drive off the  $H_2S$  and concentrating at a low temperature to the consistence of a thin syrup. If this is poured gradually and with constant stirring into a large quantity of absolute alcohol an abundant white flocculent precipitate is obtained which is a highly purified extract containing the poisonous substance, sugars, and inorganic salts. It is white and very hygroscopic, but when dried *in vacuo* over sulphuric acid is easily powdered. The yield is about 4 per cent., it is physiologically active, and was used for many of my experiments. The alcohol holds in solution more or less of the active constituent, sugars, etc., and these may be recovered by distilling off the alcohol to a small bulk and again precipitating with absolute alcohol.

From the extract the active acid may be obtained by dissolving in a small quantity of water and carefully precipitating with a saturated solution of lead acetate. The lead salt is not insoluble in acid water, and unless care is exercised in this respect much may be lost. The lead precipitate is decomposed by  $H_2S$ , filtered, the filtrate freed from  $H_2S$ , concentrated to a syrup and poured into absolute alcohol which precipitates the acid as a salt in combination with various bases present in the peas. It can be converted into a sodium salt, by dissolving in water and carefully neutralising with sodium bicarbonate which throws out the insoluble calcium and magnesium carbonates and they are filtered off. The filtrate on drying yields a white powder consisting chiefly of the sodium salt of the active acid which is very soluble in water. The acid may be obtained from it by adding slight excess hydrochloric acid and extracting with hot 85 per cent. alcohol. On allowing the alcohol to evaporate slowly it usually dries up to an amorphous glassy mass, but occasionally small quantities of acicular crystals formed. These have not been obtained as yet in sufficient quantity for a thorough chemical examination, but research in this direction is being continued.

In a former research I obtained a small quantity of the same substance, but described it as an alkaloid, the mistake arising from the presence of a trace of organic matter which gave precipitates with many of the usual alkaloidal reagents.

## EXPERIMENTS WITH THE ACTIVE PRINCIPLE AND THE EXTRACT.

*Monkeys.*

A monnet Monkey got hypodermically 0.8 grm. of the sodium salt of the active acid. In 10 minutes it had become very weak and lay down flat on its side; it could not stand, could only drag itself about, and at times its legs were stiffly stretched out as if in spasm. This lasted all day, on the following day it was merely paretic, and on the third day apparently normal.

When 2-4 grm. of the purified extract was given hypodermically the effects

varied from weakness to almost complete helplessness and spasmodic attacks. In the former case the animal sat with its back bent and its head down between its legs and looked drowsy and indifferent. If roused it moved about but had lost all its agility. When more severely affected it lay down and all its joints were flexed as if the weakened extensor muscles had been overcome by the also weakened flexors (Plate VII, fig. 1). Sometimes it developed irregular clonic and tonic spasms of groups of muscles in which the joints might be more firmly flexed. One limb might be much more affected than the others and might remain much longer affected. The effects might pass off in a few hours or might last in a modified degree for 3 or 4 days.

A peculiar feature of the action on monkeys is that marked paresis or a spasmodic attack sometimes come on suddenly when the animal is getting no lathyrus, and often a very considerable time after the last administration and when it is apparently normal. Thus a monkey which had been given nine hypodermic injections at intervals from January to July suddenly developed a severe convulsive attack on September 3rd. In January and July of the following year it got two further doses. It remained perfectly well except for a slight degree of permanent paresis of its limbs until September 20th, when it was found paralysed and moribund. Its organs were healthy and there was no gross apparent cause of death, but minute examination of the nervous system revealed extensive degenerative changes. The same delay has been observed in horses. In an outbreak in a stable of 80 horses at Liverpool the last case occurred 8 weeks after the lathyrus feeding had been stopped. It is improbable that the poison remains so long in the nervous system and there is the further fact that the animals appear to be quite or nearly quite well previously, hence the most likely explanation is that the lathyrus initiates irritative and degenerative changes in the nerve fibres and nerve cells which increase progressively and at times give rise to the paralytic convulsive attacks.

The electrical reactions of the muscles and motor nerves remained normal, and this makes it improbable that the peripheral nerves are directly affected.

#### *Rabbits.*

Rabbits with 2 grm. of the extract given hypodermically may show no effects or only some weakness in the legs which does not last long. This is not surprising, as rabbits thrive well when fed exclusively on the peas for months. One rabbit (500 grm.) became very parietic and died from gradual paralysis.

#### *Frogs.*

In frogs the sodium salt and the extract are marked muscle and nerve poisons. I obtained a small quantity of the active acid in crystals and when 0.02 grm. of this was neutralised with sodium bicarbonate and given hypodermically the frog became paralysed in 3 minutes due to an action on the brain and spinal cord. The heart went on beating for 2 hours. After 0.01 grm.

there was more or less paralysis for a few hours, and this was succeeded by tetanic spasms or greatly increased spinal reflexes. The muscles in the neighbourhood of the injection were poisoned and often irresponsive to the faradic current.

When 0.1–0.2 grm. of the extract was given the frog became more or less paralysed for some hours, and this was succeeded by increased reflexes which might last for days if the animal survived.

#### LESIONS IN THE BRAIN AND SPINAL CORD OF MONKEYS.

The cord showed degeneration of nerve fibres in all its columns and in all of about an equal degree, along with degenerative changes in the anterior and posterior nerve roots. The degeneration is very scattered and widespread, but does not involve large areas, only fibres here and there being picked out. In the brain the fibres of the corona radiata, crura cerebri, pons and medulla oblongata presented similar changes, both motor and sensory tracts being involved. In the cerebellum the degeneration was much less in degree. Some of the large nerve cells in the cortex cerebri, corpora striata, optic thalamus and cord showed all degrees of chromatolysis and in some of them the cell nucleus had disappeared. These pathological changes correspond with the symptoms observed in monkeys. The viscera were normal and healthy.

#### PREVENTION.

In the seventeenth century two Dukes of Württemberg in succession prohibited the cultivation of lathyrus in their domains, and in India and Algeria similar attempts have been made locally, but these measures have always proved unpopular and quite ineffective. Lathyrus peas are probably not more poisonous than many other kinds of peas, but poverty and primitive inefficient agriculture compel large populations in India to eat them in excess, and hence a more varied and better balanced dietary is the true preventive if it could be attained. Failing that, soaking the decorticated peas or the meal overnight in twice their weight of soft cold water and draining off the water with gentle pressure deprives them of at least one-quarter of the toxic substance. The cold water removes very little protein and no starch. Undecorticated peas part with a mere fraction of the active principle to cold water.

In a vegetarian population in times of famine and generally a lathyrus diet should not be supplemented, as it often is, by other pulses, which may be just as dangerous, but by cereals, fresh vegetables and fats, all of them, however, much more costly.

#### OTHER LEGUMINOUS SEEDS.

I have extended these observations to a number of other well-known pulses, but owing to the time and labour involved have so far only been able to overtake a few of those most widely used for human food or cattle fodder. Others, however, are being investigated.

The result is somewhat surprising, as it demonstrates that they can be as toxic as lathyrus, but like it different samples vary a good deal in toxicity. The active principle seems to be the same in all and practically the same methods of isolating it or obtaining a highly purified extract were employed.

#### *ERVUM LENS—LENTILS.*

Lentils are used as a staple food by many millions of human beings and are supposed to be one of the earliest plants reclaimed from its wild state and cultivated by man.

#### *Monkeys.*

A Bonnet monkey ( $3\frac{1}{2}$  kgm.) was fed on a diet of lentils cooked by steaming and heavily flavoured with orange juice. It ate about 120 gm. per day and some milk and fruit were given in addition.

In 3 days the left arm was paretic and held somewhat adducted and bent at the elbow. During the next 6 weeks it gradually lost its wonted agility, its muscles became weak and tremulous, it climbed slowly and circumspectly and sometimes fell. Latterly it often sat for long periods with its head down between its legs and its back bent, and having slight general muscular tremors, the whole picture being very reminiscent of an advanced case of paralysis agitans (Fig. 3). It ate well and put on weight but varied a good deal in alertness. On the 49th day it became much worse and moved about only on all fours; the flexor and extensor muscles did not act in unison and this gave it a very jerky, slow gait. There were coarse muscular tremors. It died on the 52nd day from gradually deepening general paralysis. Its organs were all healthy, but on microscopic examination the brain and spinal cord showed similar degenerative changes to those of lathyrus poisoning. Judged by this feeding experiment this sample of lentils proved more poisonous than most samples of lathyrus peas which I have used on monkeys.

A Bonnet monkey was given hypodermically 4 gm. of a purified extract. It sat crouched up all day and very inactive. Two days later 5 gm. were given and in 10 minutes it could not support itself upright and was extremely feeble. A day later 2 gm. were given and in a few minutes it was very paretic. In half an hour it had clonic spasms of the right arm and head and 10 minutes later this passed into general clonic convulsions with all its joints flexed, exactly as has been described with lathyrus (Fig. 2). These gradually passed off and 6 hours later it was moving about but with its joints still slightly flexed. Next day its arms were extremely weak and the digits flexed, but, on the day following, it had regained most of its normal activity.

#### *Rabbits.*

A rabbit which received 4 gm. of the same extract hypodermically showed no symptoms whatever.

*Frogs.*

A very large dose of the crystalline active acid (0.07 grm. neutralised with sodium bicarbonate) caused complete paralysis in a large frog almost at once. It was examined an hour later. The heart had stopped and most of the muscles did not react to electric stimulation. Smaller doses caused depression followed by increased reflexes.

The purified extract had a similar action according as the dose was large or small.

*PISUM SATIVUM*—THE COMMON CULTIVATED PEA.

Peas are imported into this country from many parts of the world and when fully ripe may have either a yellow or green colour. Both kinds were examined. No observations were made with fresh green peas. The yellow peas used were imported from Chile and Calcutta for ordinary sale here.

*Monkeys.*

A monkey (3.2 kgm.) was fed on "split" peas cooked by steaming, along with some fruit and milk daily. It ate about 100 grm. per day. After a month no toxic symptoms were visible. It was then given in addition water in which 200 grm. of the peas had been soaked for 24 hours. The effect of the increase of dose was very marked. After each meal it became inactive and drowsy and sat very much bent down for 2 or 3 hours. It was supplied with such water for 5 days in succession when it had become very paretic and dragged its right leg. It remained in this condition more or less till the 54th day when it suddenly became much weaker (Fig. 6) and on the 55th day was almost completely helpless (Fig. 4), but drank milk freely. On the 56th day it died from gradually deepening paralysis.

A somewhat larger monkey received 4 grm. of a highly purified extract hypodermically. In a few minutes it became less active and remained so all day, getting gradually worse. On the 2nd day it was more paretic still, hardly moving unless roused, and sat with bent back and its chest resting on its legs. If roused it moved stiffly and with bent knees. It continued much in this condition for 5 days longer and died on the 7th day from gradually increasing paralysis.

The pathological changes in the brain and spinal cord were similar to those already described as occurring with lathyrus. The viscera were all healthy.

*Frogs.*

When 0.05 grm. of the pure crystalline acid (neutralised with sodium bicarbonate) was injected under the skin of the back the frog became completely paralysed and insensitive to stimuli in 10 minutes. Five hours later the heart

was still beating feebly, but the back muscles were irresponsive to the faradic current.

With 2 ctgrm. the initial paralysis was followed in 3 hours by tonic spasm of the limbs. It remained paretic with increased reflexes but had recovered fully on the 3rd day.

The purified extract given in considerably larger doses had a similar action.

Dry green peas are a variety known as the "blue" pea, owing to its bluish green colour. They are grown to some extent in England but are mostly imported from the Continent. No experiments were made with them on monkeys, but their chemistry and their action of frogs and rabbits differed in no way from the yellow pea.

#### *SOYA HISPIDA* (GLYCINE HISPIDA)—THE SOYA BEAN.

This is a medium-sized bean with a white shiny seed-coat. In the Far East it is next to rice the most important vegetable food, being eaten by all classes and in some form or other almost at every meal. It is very rich in protein (34 per cent.) and in fat (20 per cent.), is therefore very highly nutritious and forms an excellent complement to rice in a national dietary. It is eaten to some extent boiled like other beans, but in China and Japan is manufactured by elaborate processes into a great variety of products (so-called "cheese" of different kinds) which are important foods. Soy sauce is also made from it. It is imported into this country in large quantities, the fat (a thickish yellow oil) is removed by solvents and the meal is then used as a cattle food.

For the most part it has proved highly satisfactory as a cattle food, but from time to time cases of poisoning have occurred from its use, sometimes on a considerable scale, and have been reported in the veterinary journals. For my experiments the defatted meal was used.

#### *Monkeys.*

A monkey was fed on 120 gm. per day with some fruit in addition. For a fortnight there was no change and then it was given drinking water in which 100 gm. of the meal had been soaked. This addition was followed at once by marked paresis, the monkey became much less active and often sat bent up. When 200 gm. of the soya meal was macerated in its drinking water it became very weak and only moved about slowly and carefully with no spring in its legs and its back and joints bent. It was drowsy and somnolent (Fig. 5), but by next day had always partially recovered. The 120 gm. *per os* sufficed merely to keep it very inactive and feeble, but any considerable addition in its drinking water at once intensified its symptoms. The experiment was stopped on the 31st day; it was fed on bread and milk and fruit, the paresis gradually passed off, and in a fortnight it seemed perfectly normal again.

A Rhesus monkey was given 0.7 gm. of the sodium salt of the active acid hypodermically. In a few minutes it lay down and all day was hardly able



to sit up. Next day it moved about but was not able to climb; on the 3rd day it was running about, climbed very slowly, and its muscles were weak. On the 4th day it was about normal except that its toes were much flexed.

In this monkey the motor tracts seemed to be the only part of the nervous system affected. There was no drowsiness or somnolence and it remained quite alert.

A month later the same monkey was given 5 grm. of a purified extract and remained very paretic for 4 days.

#### *Rabbits.*

Rabbits showed no symptoms with 4 grm. of the purified extract. When 1 grm. of the sodium salt was given the animal became very feeble and had marked weakness in its hind legs.

#### *Frogs.*

I obtained a small amount of the active acid in well-formed acicular crystals. When 0.03 grm. neutralised with sodium bicarbonate was administered to a large frog hypodermically it became paretic at once and 35 minutes later its right leg was in tetanic spasm while the rest of its body was very limp. In an hour it had severe general tetanus and an hour later was killed. The heart was still beating, the back muscles did not respond to electric stimulation, but the other muscles, the spinal cord and the motor nerves responded. Larger doses rapidly caused complete paralysis and stopped the heart. The muscles were usually more or less excitable to the faradic current as they had not had time to be completely paralysed. With 0.01 grm. there was marked paresis and 45 minutes later violent tetanus. Next day the reflexes were increased, but by evening had subsided to normal. The purified extract in 0.1–0.2 grm. doses had a similar effect.

#### *VICIA SATIVA—TARES.*

The whole plant is very largely grown for cattle food. The peas as found in commerce vary a good deal in size and have a black seed-coat. For these experiments Russian and Swedish tares were used.

#### *Monkeys.*

A Bonnet monkey was given 0.95 grm. of the sodium salt of the active acid hypodermically. In 2 minutes it lay down on its side and for some hours was hardly able to stand or walk. The right leg was often extended in spasm, the other limbs remaining very limp and feeble. Seven hours later it was able to walk stiffly and feebly on all fours. Next day it was very paretic and on the 3rd day had regained much of its normal condition.

The same monkey was given hypodermically 4 grm. of the purified extract. This caused marked general weakness, but especially of the right arm, inversion of the feet and slight flexion of the joints, but it was able to move about. The effect lasted 24 hours.

### *Frogs.*

A dose of 0.01 grm. of the crystalline acid given as a sodium salt caused some depression followed by an increase of reflexes. Larger doses (2–5 ctm.) proved to be rapidly acting nerve and muscle poisons.

A dose of 0.2 grm. of the extract hypodermically caused complete paralysis in a few minutes. There was no eye reflex and no response to stimulation. On examination some hours later the heart had ceased to beat and the muscles along the back where the injection had been made did not respond to the faradic current.

Smaller doses caused depression for some hours according to the amount given, and this was followed by increase in the spinal reflexes lasting for several days.

### *Rabbits.*

A rabbit (650 grm.) was not visibly affected by 4 grm. of the purified extract.

Another rabbit (1300 grm.) received hypodermically 1.2 grm. of the sodium salt. It became paretic and lay down on its belly, owing to weakness of the legs. Two hours later it had practically recovered.

### *ERVUM ERVILIA*—THE BITTER VETCH.

The peas are small, triangular in shape, and the seed-coat has a light brown colour. They seem to have been used by the ancient Greeks as food, and Proust states that the Kabyles so use them. The whole plant is grown in Algeria as fodder and there are occasional accounts of poisoning of animals by it and by the seeds.

### *Monkeys.*

A monkey (2 kg.) was given hypodermically 3 grm. of a highly purified extract made from the peas. The only effect visible was some degree of paresis. Next day it received 4 grm. and in half an hour was unable to stand or climb or grip a bar. It continued weak and much less active for some days afterwards, but gradually recovered completely its normal activity.

### *Rabbits.*

In rabbits 4 grm. of the extract had no apparent action.

*Frogs.*

A dose of 0.2 grm. of the extract brought on complete paralysis in half an hour. Five hours later it was sitting up, very paretic and sluggish but with increased spinal reflexes. Next day its reflexes were much increased, almost to tetanus, and this increase continued during the next 4 days when it died.

With 0.1 grm. the depression stage was not nearly so marked and was succeeded by more or less exaggerated reflexes for some days.

I succeeded in obtaining a small amount of the active acid in pure acicular crystals. A dose of 0.05 grm. neutralised with sodium bicarbonate was injected under the skin of the back of a large frog. In 10 minutes it was absolutely paralysed, the brain and spinal cord were completely out of action. The muscles of the back and trunk and thighs were very white, in rigor mortis, and irresponsive to electric stimulation.

*CAJANUS INDICUS*—THE PIGEON PEA.

The plant is very extensively grown in Africa and in the East and West Indies. The peas are small with a shiny orange-brown seed-coat. In India they are known as arhar (urhur) and are largely consumed, made into cakes, or boiled with water and spiced, or cooked in hot sand and eaten dry or with salt or oil. In Jamaica they are cooked fresh or dried, and are also used to feed pigeons.

No experiments were made on monkeys but a highly purified extract given hypodermically to rabbits and frogs had the same action as has been described as occurring with other peas.

## COMMENTARY.

In all these seeds the poisonous body is an acid and apparently the same acid in all. In large doses its action on frogs is always qualitatively the same, namely, depression of the brain and spinal cord, succeeded, if the dose be not lethal, by more or less increase of spinal reflexes, and the motor nerves are not paralysed. The muscles are also poisoned, more especially in the area of the injection but also more widely through the blood. With very small doses the depression stage may be hardly noticeable and there is an equally slight increase of spinal reflexes.

Rabbits (herbivora) are very insusceptible, but with large doses show a certain amount of weakness of the legs.

In monkeys after a large or frequently repeated smaller dose, the symptoms broadly are those of depression and irritation of the brain and cord, but in detail they vary somewhat. Sometimes drowsiness and lethargy are very prominent, sometimes weak tremors or coarse jerking of muscles, or clonic or tonic convulsions of groups of muscles in rapid succession and involving

the whole body, sometimes a shaking palsy like paralysis agitans. But always there is marked paresis of the motor tracts and muscular weakness. The more powerful flexors and adductors overcome the extensors and abductors giving rise to flexion of the joints and adduction of the limbs. One limb is often earlier and much more affected than the others. The chief effect therefore is on the cerebrum (fore-brain, motor areas and basal ganglia) and the spinal cord, but it is not always the same part which is chiefly affected and hence the considerable variation in symptoms. This is closely comparable to what is seen in man in encephalitis lethargica and in severe chronic mercurial poisoning, and for the same reason in all three, namely, that different areas in the cerebrum may be affected and these areas in varying degree.

A detailed description of the pathological changes which were found in the nervous system of the monkeys which died will be published later and meantime it is sufficient to say that all the peas examined gave rise in monkeys to degeneration of nerve cells and fibres in the brain and spinal cord and that this fully explains the symptoms.

In using fifteen different lots of lathyrus I have found that they vary very greatly in toxicity, and this is probably true also of other peas and accounts no doubt for the cases of poisoning which have occurred occasionally among farm stock with soya meal, and with the bitter vetch and lathyrus itself. My feeding experiment on a monkey with lentils shows that an individual sample of these may also be very poisonous.

The universal experience of mankind is proof that pulses in moderation form pleasant, cheap and nourishing food, and so far lathyrism is the only disease which is known to arise from their use. Short of causing a disease easily recognised clinically the consumption of pulses as the chief part of a dietary may conceivably give rise to some deterioration of health or energy, but I have searched medical literature in vain for any indication of this. It could only occur in communities which are strictly or mainly vegetarian and probably the populations concerned have settled the matter for themselves in normal (non-famine) circumstances by use and wont more satisfactorily than science could do. An interesting question arises as to whether the presence of a nervine stimulant in leguminous seeds has had any influence in determining their almost universal use as a food by mankind. An analogous case is that of caffeine, the presence of which in many different plants has led to a general use of these in widely separated regions of the globe from time immemorial. Like opium, coca, tobacco and many other drugs of the same kind these leguminous seeds may be classed as *stimulant-narcotics*, that is, stimulants to the nervous system in small doses and narcotic in large doses, with the difference that they are capable of producing much more readily organic degenerative changes in the central nervous system. Fortunately, however, to do this very large amounts are required. Lathyros, the Greek name for vetchlings, is stated to have its root in *θούρος* (exciting, impetuous), and Cicer, as the Romans called the chick-pea (*C. arietinum*, the Anglo-Indian

*gram*) is derived from the Greek *κίκυς* (force, vigour), its ordinary Greek name *ἐπεβίρωτος* having the same significance. Greek and Roman writers on agriculture and natural history and diet refer to and sometimes lay stress on the stimulant properties of pulses both for man and domestic animals, but modern authorities on dietetics seem to have lost sight of this and discuss them only in terms of protein, fat, carbohydrate, calories and vitamins.

#### EXPLANATION OF PLATE VII.

Fig. 1. Lathyrus: paralysis and flexion of joints.

Fig. 2. Lentils: hypodermic injection of active substance; paralysis, flexion of joints and clonic spasms.

Fig. 3. Lentils: feeding; paresis and somnolence.

Fig. 4. Peas: feeding; paralysis.

Fig. 5. Soya: feeding; paresis and somnolence.

Fig. 6. Peas: feeding; paresis and somnolence.

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# MORTALITY FROM CANCER ACCORDING TO SITE IN THE COUNTIES OF SCOTLAND, 1923-8.

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As Scotland is the only home country for which information is published on the regional distribution of the mortality from cancer according to site, it seemed of interest to examine whether a particular site, or group of sites, had any special geographical location. With this object in view the present investigation was undertaken for the period 1923-8.

The sites of cancer as classified for each county in the *Annual Reports of the Registrar General for Scotland* are (1) buccal cavity; (2) stomach and liver; (3) peritoneum, intestine, rectum; (4) female genital organs; (5) breast; (6) skin; (7) other organs.

The total deaths only and not the deaths at specified ages were published for these sites. It was assumed, however, that all these deaths occurred after age 35 years. This assumption will introduce a negligible error, since 97 per cent. of the total cancer deaths occur after this age. Since the deaths were not given for specified ages it was necessary to adjust the crude death-rate at ages 35 years and upwards in each county—the deaths at age 35 years and over divided by the corresponding population, so as to eliminate the effects of the differences in the age and the sex constitution of the various populations. This procedure is absolutely essential in dealing with a disease like cancer which mainly affects the higher age periods. The method was that known as "indirect standardisation."

Possessing the requisite data in a suitable form we are now in a position to ascertain the magnitude of the standardised death-rates from cancer of particular sites and also the range of their variability in the counties of Scotland (see Table I). The essential facts are present in Table I in which Col. 1 contains the mean or average standardised death-rate for the particular site, or group of sites, in the counties; Col. 2 indicates the average variability in the mortality rates when the variability of each county is measured from the mean death-rate given in the previous column. Col. 3 enables us to compare the amount of dispersion or scatter in the rates of mortality in the different sites because the values of the standard deviation are expressed as percentages of the corresponding mean death-rates, the formula for the "Coefficient of Variation," in the present instance, being

$$\frac{\text{Standard deviation}}{\text{Mean death-rate}} \times 100.$$

<sup>1</sup> Working for the Medical Research Council.



Table I. *Showing the standardised death-rate per 1000 at age 35 years and upwards and the variability of cancer of particular sites in counties of Scotland, 1923-8.*

Site	Standard death-rate		Standard deviation		Coefficient of variability	
	Persons	Females	Persons	Females	Persons	Females
Buccal cavity ... ..	0.180	—	0.0388	—	21.6	—
Stomach and liver ... ..	1.066	—	0.2272	—	21.3	—
Peritoneum, intestine, rectum	0.738	—	0.1594	—	21.6	—
Breast... ..	—	0.544	—	0.1192	—	21.9
Skin ... ..	0.049	—	0.0277	—	56.5	—
Female genital organs ... ..	—	0.546	—	0.1359	—	24.9
Other organs ... ..	0.599	—	0.1328	—	22.1	—

As was to be expected, the greater part of the total mortality from cancer occurs in the group stomach and liver, the death-rate being 1.066 per 1000, or six times greater than that for the buccal cavity. In the two sites common to women, the genital organs and breast, the rates of mortality are almost equal, being 0.546 per 1000 for the former and 0.544 for the latter. The death-rate from breast cancer, as given in this table, overstates the actual facts to a very small degree, because the deaths as published for each county refer to the males and females combined, but in calculating the death-rate the female population only was used. If the male population had been included in the denominator of the fraction

$$\frac{\text{Deaths from breast cancer}}{\text{Population for persons}},$$

then the prevalence of cancer in this site would have been considerably underestimated. That the inclusion of the male deaths will not be a factor of much importance will be readily understood when it is pointed out that in the whole of Scotland, during the period 1923-8, the male deaths in this site formed less than 1 per cent. of the total number from breast cancer.

When the relative variability of the mortality in different sites is examined it will be observed that it is remarkably constant. The only exception is skin cancer, for which the index is 57 per cent.; the fluctuations in all the other sites lie between 21 per cent. and 25 per cent. No undue importance may be attached to the variation in the mortality from skin cancer, as the amount of cancer in this site is relatively small, being less than 2 per cent. of the total deaths, and the highest standardised death-rate, 0.132 per 1000 in Nairn, was based on a total of four deaths in six years.

We may approach the problem of the geographical distribution of the mortality according to site from another angle by comparing the expected number of total deaths with the actual or observed. The expected deaths in any one county are those which would occur if the male and female population in that county had experienced the rates of mortality for the separate sexes at the various age periods in the whole country. The difference between the actual and expected number of deaths when tested against the standard error

of the difference will indicate the degree of importance attaching to the amount of cancer of any particular site present in any one county. The standard error of the difference may, for all practical purposes, be regarded as measured by  $\sqrt{E}$ , where  $E$  is the expected number of deaths. Unless the difference exceeds at least twice this criterion it cannot be regarded as of any statistical importance. Applying this test it was found that, for the inaccessible sites of cancer, the recorded number of deaths significantly exceeded the expected in certain counties, but these were mainly counties in which medical skill and the probability of better provision as regards mechanical facilities for diagnosis existed. For instance, in Lanarkshire, containing as it does the city of Glasgow, the actual number of deaths in the group cancer of stomach and liver considerably exceeded the expected; the recorded deaths were 4172, the expected 3419, a difference of 753, which is at least twelve times greater than its standard error. The other counties which had an excess of actual

Table II. *Showing the distribution of deaths from cancer of the breast in the counties of Scotland, 1923-8.*

Counties	Actual	Expected	Diff.	S.E.	Diff. S.E.
Aberdeen ... ..	227	237	-10	15.39	0.65
Argyll ... ..	56	73	-17	8.54	1.99
Ayr ... ..	206	197	+ 9	14.03	0.64
Banff ... ..	37	46	- 9	6.78	1.33
Berwick ... ..	32	26	+ 6	5.10	1.18
Bute ... ..	17	32	-15	5.66	2.65
Caithness ... ..	24	27	- 3	5.20	0.58
Clackmannan ... ..	21	24	- 3	4.90	0.61
Dumbarton ... ..	95	93	+ 2	9.64	0.20
Dumfries ... ..	60	62	- 2	7.87	0.25
East Lothian ... ..	39	34	+ 5	5.83	0.86
Fife ... ..	198	200	- 2	14.14	0.14
Forfar ... ..	222	235	-13	15.33	0.85
Inverness ... ..	75	81	- 6	9.00	0.67
Kincardine ... ..	33	32	+ 1	5.66	0.18
Kinross ... ..	6	7	- 1	2.65	0.38
Kirkcudbright ... ..	33	33	0	5.74	—
Lanark ... ..	902	875	+27	29.58	0.91
Midlothian ... ..	423	392	+31	19.80	1.57
Moray ... ..	36	38	- 2	6.16	0.32
Nairn ... ..	6	9	- 3	3.00	1.00
Orkney ... ..	15	26	-11	5.10	2.16
Peebles ... ..	6	15	- 9	3.87	2.33
Perth ... ..	136	120	+16	10.95	1.46
Renfrew ... ..	223	185	+38	13.60	2.79
Ross and Cromarty... ..	60	70	-10	8.37	1.19
Roxburgh ... ..	55	43	+12	6.56	1.83
Selkirk ... ..	21	21	0	4.58	—
Shetland ... ..	18	30	-12	5.48	2.19
Stirling ... ..	89	98	- 9	9.89	0.91
Sutherland ... ..	15	19	- 4	4.36	0.92
West Lothian ... ..	42	40	+ 2	6.32	0.32
Wigtown ... ..	23	26	- 3	5.10	0.59

deaths as compared with the expected deaths were West Lothian, Midlothian, Renfrew and Forfar, but the disparity was not so well defined as in Lanarkshire. In the Highland counties the position was reversed—the indices were significantly negative, the ratio in Orkney being -4.1, Shetland -4.5,

Inverness -4.2, Aberdeen -3.9, Ross and Cromarty -3.9, Argyll -3.6, and Sutherland -3.6. It will be observed that the county of Aberdeen lies within the negative category, and there is no obvious reason for the deficiency in the amount of cancer of the stomach in that county.

As, with the possible exception of lingual cancer, errors are less likely to occur in the diagnosis of cancer of the breast than in any other site and, as cancer of the buccal cavity can be equally certified in town or country, the distribution of the actual and expected number of deaths occurring in these two sites during the period 1923-8 are shown for the individual counties in Tables II and III.

Table III. *Showing the distribution of deaths from cancer of the buccal cavity in the counties of Scotland, 1923-8.*

Counties	Actual	Expected	Diff.	S.E.	Diff. S.E.
Aberdeen ... ..	125	147	-22	12.12	1.81
Argyll ... ..	42	49	-7	7.0	1.00
Ayr ... ..	99	122	-23	11.04	2.08
Banff ... ..	26	31	-5	5.57	0.89
Berwick ... ..	11	18	-7	4.24	1.65
Bute ... ..	10	17	-7	4.12	1.70
Caithness ... ..	19	19	0	4.36	—
Clackmannan ... ..	15	15	0	3.87	—
Dumbarton ... ..	60	59	+1	7.68	0.13
Dumfries ... ..	34	39	-5	6.24	0.80
East Lothian ... ..	34	21	+13	4.58	2.84
Fife ... ..	116	125	-9	11.18	0.81
Forfar ... ..	159	129	+30	11.36	2.64
Inverness ... ..	52	56	-4	7.48	0.53
Kincardine ... ..	18	21	-3	4.58	0.66
Kinross ... ..	3	5	-2	2.24	0.89
Kirkcudbright ... ..	22	21	+1	4.58	0.22
Lanark ... ..	610	566	+44	23.79	1.85
Midlothian ... ..	268	222	+46	14.90	3.09
Moray ... ..	22	24	-2	4.90	0.41
Nairn ... ..	6	6	0	2.45	—
Orkney ... ..	13	18	-5	4.24	1.18
Peebles ... ..	6	8	-2	2.83	0.70
Perth ... ..	78	73	+5	8.54	0.59
Renfrew ... ..	115	115	0	10.72	—
Ross and Cromarty... ..	35	49	-14	7.00	2.00
Roxburgh ... ..	24	26	-2	5.10	0.39
Selkirk ... ..	9	13	-4	3.61	1.11
Shetland ... ..	12	17	-5	4.12	1.21
Stirling ... ..	54	66	-12	8.12	1.48
Sutherland ... ..	17	15	+2	3.87	0.52
West Lothian ... ..	24	30	-6	5.48	1.10
Wigtown ... ..	13	17	-4	4.12	0.97

#### BREAST CANCER.

One would naturally expect to find in the case of mammary cancer the highest mortality in the Highland counties where promptitude of medical attention and facilities for surgical treatment might be expected to be more deficient than in the Lowlands, but there is, as will be seen from Table II, no defined localisation. If anything, the prevailing tendency is towards a deficiency in the amount of breast cancer in the Highlands, but the deficit is not equally marked in the individual counties constituting the group. In

only one county in Scotland—Renfrew—is the difference between the actual and expected values possibly significant. If the standard errors of the differences were normally distributed we should expect to find that the indices in Col. 5 would be grouped as follows:

*A.* Twenty-three counties showing a difference of less than once the standard error.

*B.* Nine counties showing a difference greater than once but less than twice the standard error.

*C.* One county showing a difference greater than twice but less than three times the standard error.

When the ratios in Col. 5 are assigned to these categories the distribution is 20 in *A*, 8 in *B* and 5 in *C*. Although there is an apparently large discrepancy between the actual and expected totals, *i.e.* 5 and 1 respectively in Group *C*, it is not of real importance, as the indices are barely outside the limits of significance, and furthermore, with the exception of the case of Renfrew, they are associated with counties containing relatively small populations.

#### CANCER OF THE BUCCAL CAVITY.

As will be observed from the statistics in Table III there is really no specific localisation of the deaths in this site. There is only one county—Midlothian—in which more deaths are enumerated than would be expected on the basis of the mortality rates in the whole country. In all the other counties there is no satisfactory evidence to indicate that the amount of cancer deviates appreciably from the normal standard.

#### BIRTH-RATE AND CANCER AMONGST WOMEN.

In the *Annual Report of the Registrar General for England and Wales* for 1913, Dr Stevenson has shown that during the three years 1911–13 the mortality from breast cancer amongst unmarried women was 45 per cent. higher than amongst the married, but as regards uterine cancer the position was almost exactly reversed. It would thus appear that the presence or absence of childbearing is a factor of some importance in influencing the mortality from two forms of cancer amongst women. To what extent are they dependent? For a reliable answer to this question we are indebted mainly to the work of Prof. Greenwood and his colleagues<sup>1</sup>. In his analysis of the data for 39 administrative counties in England and Wales he found that the birth-rate during the period 1901–3, based on the number of married women between the ages of 15 and 45 in each county, and the mortality from breast cancer at ages over 25 in the period 1911–20 were correlated to the extent of  $-0.514 \pm 0.079$ . In other words a low birth-rate was reflected, though not absolutely, in a high mortality from breast cancer. As regards the influence of fertility on the presence of uterine cancer there was little or no relationship, as the coefficient, although negative, was within the error of random sampling.

<sup>1</sup> *League of Nations. Health Organisation Sub-Committee on Cancer. C.H. 333, 1.*

His results, in the case of the breast cancer and fertility, were confirmed by investigators in two other countries, Italy and Holland, although their conclusions were based on a rather small series of observations.

The position, as regards uterine cancer and fertility in Italy and Holland, particularly in Holland, was quite different from that in England, as, in Holland, the birth-rate was more highly correlated with uterine cancer than with breast cancer. In view of these results it was of interest to examine the degree of relationship exhibited by the Scottish data. For this purpose the birth-rate at each of the two triennial periods, (1) 1900-2 and (2) 1910-12, expressed in terms of the married women 15-45 in the individual counties, was correlated with the standardised mortality rates from breast cancer and uterine cancer respectively during the period 1923-8. The periods 1900-2 and 1910-12 were selected from the fertility standpoint because the majority of the cancer deaths occurring in the two sites during 1923-8 must inevitably have occurred amongst women who were mothers during these two triennia. When the necessary coefficients were calculated it was found (see Table IV)

Table IV. *Showing (1) the correlation coefficient between the incidence of cancer of special sites and fertility in the counties of Scotland, (2) similar results for other countries.*

(1) SCOTLAND. Standardised death-rate at age 35 years and upwards for the period 1923-8.

	Cancer of breast	Cancer of genital organs (female)
Birth-rate (based on married women 15-45) for the period 1900-2 ... ..	$r = +0.096 \pm 0.116$	$r = -0.092 \pm 0.116$
Birth-rate (based on married women 15-45) for the period 1910-12 ... ..	$r = +0.103 \pm 0.116$	$r = -0.099 \pm 0.116$
Decline in birth-rate in 1910-12 as compared with 1900-2 ... ..	$r = -0.113 \pm 0.116$	$r = -0.008 \pm 0.117$
Proportion of unmarried in female population between the ages of 30 and 45 years, 1911 census ... ..	$r = -0.434 \pm 0.096$	$r = -0.406 \pm 0.098$

(2) OTHER COUNTRIES.

England and Wales (39 adm. counties)* ...	$r = -0.514 \pm 0.079$	$r = -0.142 \pm 0.106$
Italy (16 regions)† ... ..	$r = -0.580 \pm 0.112$	$r = -0.430 \pm 0.200$
Holland (11 provinces)‡ ... ..	$r = -0.507 \pm 0.151$	$r = -0.538 \pm 0.144$

\* Mean birth-rate 1901-3 per 1000 married women ages 15-45 and cancer 1911-20 ages 25 and over.

† Cancer mortality all ages over 20, 1919-21. Legitimate births 1919-21 in terms of married women 15-45 (June, 1911).

‡ Mean birth-rate 1900-4 per 1000 married women under 50 and cancer 1913-22 all ages.

that there was no relationship between the amount of breast cancer and the previous size of the birth-rate. The values obtained were positive, as compared with the negative coefficients found in the other countries, but they were statistically unimportant. A further test was applied. The mortality from breast cancer was correlated with (1) the decline in the birth-rate in each county between 1900-2 and 1910-12, but no direct or indirect relationship was found to exist; (2) with the proportion of single women in the female population between the ages of 30 and 45 years in each county at the 1911 census. The age limits 30-45 years were selected on the assumption that the

unmarried population in this age group contributed largely to the number of spinsters exposed to risk of cancer in later life. The coefficient of correlation in this instance was  $-0.434 \pm 0.096$ . This result, although contrary to expectation, was confirmed when the mortality from breast cancer was correlated with the proportion of single women aged 45 years and upwards in the total female population at this period of life at the 1921 census. The coefficient was  $-0.404 \pm 0.096$ . In view of the higher mortality from breast cancer amongst unmarried than married women as recorded by Dr Stevenson in England and Wales one would have expected a positive and not a negative answer. The coefficients were also evaluated between uterine cancer and the same factors, but no tangible relationship was demonstrated except in one instance—the proportion of unmarried was significantly correlated in a negative direction with the mortality from uterine cancer. If the absence of childbearing (as measured by the proportion of unmarried in the population) is reflected in a diminution of uterine cancer as the figures for Scotland seem to suggest, then it would appear logical to assume that the impairment of the physiological functioning which often occurs at birth would find expression in a positive association between uterine cancer and fertility. The correlation coefficients between the two variables as found in each of the four countries lend no support to this deduction.

From this survey of the distribution of the mortality from cancer according to site in the counties of Scotland during the six years 1923–8, the following is a fair representation of the facts.

#### CONCLUSIONS.

1. With the exception of skin cancer the relative variability of the mortality in the other sites, or group of sites, is remarkably constant in Scotland as the coefficients of variation fall within a range of 21 to 25 per cent. (see Table I).

2. The counties possessing the best equipped hospitals have a greater amount of inaccessible cancer than might be expected to occur. In Lanarkshire, for example, the actual deaths from cancer of the stomach and liver numbered 4172, the expected 3419—a difference of 753, which is approximately thirteen times greater than its standard error.

3. As regards the two accessible sites—breast and buccal cavity—there is no defined localisation in the mortality, as the number of deaths in each county, with possibly two exceptions, Renfrew for breast cancer, Midlothian for buccal cavity, approximately follow a normal distribution.

4. There is no evidence that the decline in the Scottish birth-rate in the individual counties has had any influence in the incidence of either breast or uterine cancer, as the coefficients of correlation were statistically insignificant.



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